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Insuficiência adrenal relativa em choque hemorrágico- estudo experimental acerca dos seus mecanismos fisiopatológicos em rato (*Rattus norvegicus*)

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“A man who has been through bitter experiences and travelled far enjoys even his sufferings after a time”
"And what he greatly thought, he nobly dared."

Homer, Odissey, V. century b.C.

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Nuno Manuel Félix
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Resumo

Título da Tese: Insuficiência adrenal relativa em choque hemorrágico- estudo experimental acerca dos seus mecanismos fisiopatológicos em rato (*Rattus norvegicus*)

Resumo:

O Choque Hemorrágico (CH) pode complicar-se por "insuficiência adrenal relativa" (IAR). Neste estudo avaliou-se a contribuição do etomidato e apoptose da adrenal na IAR pós-CH. Ratos machos Wistar foram divididos em 4 grupos: G0 (anestesia geral com isoflurano e ventilação mecânica, 16 ratos), G1 (como G0 mas com buprenorfina, 16 ratos), G2 (como G1 mas com CH, 16 ratos) e G3 (como G2 mas com etomidato antes do CH, 16 ratos). CH induzido por recolha de sangue (30% volume sanguíneo). Resuscitação aos 90 minutos pós-CH, consistindo no sangue recolhido associado a soro fisiológico. Aos 0, 90, 150 and 240 minutos após início do CH, mediram-se gasimetria e níveis plasmáticos de hormona adrenocorticotrópica, corticosterona, TNF-α, IL6, IL10 e por RT-PCR, *TNF-α*, *IL6 e IL10*. Apoptose avaliada por TUNEL e caspase-3 (immunofluorescência); necrose por escala específica. G1 apresentou taxa de apoptose mais elevada que G0. G2 e G3 tiveram níveis mais elevados de apoptose e necrose que outros grupos. Comparado com G2, G3 apresentou níveis de corticosterona compativeis com IAR e níveis mais baixos de PO_2, PO_2/FiO_2, BE, bicarbonato, apoptose e necrose, e mais altos das citoquinas e *TNF- α*.IAR foi associada a etomidato mas não a apoptose neste modelo.

Palavras-chave:

Hemorrhagic; shock; rat; adrenal; insufficiency; etomidate; apoptosis; mRNA; cytokine; buprenoprhine

Abstract

Title of the Thesis: Critical Illness-Related Corticosteroid Insufficiency in Hemorrhagic Shock- an experimental study about its pathophysiologic mechanisms in a rat model (*Rattus norvegicus*)

Abstract:

Hemorrhagic Shock (HS) can be complicated by critical illness-related corticosteroid insufficiency (CIRCI). This study evaluated if etomidate and adrenal gland apoptosis contributed to HS-associated CIRCI. Male Wistar rats were divided in 4 groups: G0 (general anesthesia with isoflurane and mechanical ventilation, 16 rats), G1 (similar to G0, but with buprenorphine, 16 rats), G2 (similar to G1 but with HS, 16 rats) and G3 (similar to G2 but with etomidate before HS, 16 rats). HS was induced by collecting 30% of the blood volume and resuscitation (90 minutes later) consisted in the collected blood and normal saline. Blood gas analysis, adrenocorticotropic hormone, corticosterone, TNF-α, IL6, IL10 and *TNF-α*, *IL6* and *IL10* mRNA quantified by RT-PCR were determined at 0, 90, 150 and 240 minutes post-HS induction. Apoptosis and necrosis were determined by TUNEL and active caspase-3 immunofluorescence and a standardized necrosis score, respectively. G1 had higher adrenal apoptosis than G0. G2 and G3 had significantly higher levels of apoptosis and necrosis. G3 had significantly lower levels of corticosterone (compatible with CIRCI), PO_2, PO_2/FiO_2, BE, bicarbonate, apoptosis and necrosis score and higher levels of cytokines and *TNF-α* than G2. Etomidate but not apoptosis was associated to CIRCI in this HS model.

Keywords:

Hemorrhagic; shock; rat; adrenal; insufficiency; etomidate; apoptosis; mRNA; cytokine; buprenorphine

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11β-HSD1-11β-hydroxysteroid dehydrogenase type 1
11β-HSD2-11β-hydroxysteroid dehydrogenase type 2
ACTH- Adrenocorticotropic hormone
ADP-Adenosine diphosphate
AIF- Apoptosis-inducing factor
ALI-Acute lung injury
ANF-Atrial natriuretic factor
ANP-Acute necrotizing pancreatitis
AP-1-Activator protein 1
ARDS-Acute respiratory distress syndrome
ATP-Adenosine triphosphate
AVP-Arginine vasopressine
BE-Base excess
Bcl2-Beta cell lymphoma 2
Bcl-XL-Beta cell lymphoma extra large
CaO_2-Arterial oxygen content
CARS-Compensatory anti-inflammatory response
CAT-Catalase
CBG-Corticosteroid-binding globulin
CI-Cardiac index
CNS-Central Nervous System
CO-Cardiac Output
COX2- Cyclooxygenase 2
CLP-Cecal Ligation Puncture
CIRCI- Critical illness-related corticosteroid insufficiency
CNA- Circulating nucleic acids
CRH-Corticotrophin-releasing hormone
CS-Corticosterone
DAMPs-Damage associated molecular patterns
DHEA- Dehydroepiandrosterone
DHEAS- Dehydroepiandrosterone sulfate
DNA-Deoxyribonucleic acid
DO_2-Oxygen delivery (to the tissues)
ERK 1-Extracellular signal-regulated kinase 1
ERK 2-Extracellular signal-regulated kinase 2

GC-Glucocorticoid

GCR-Glucocorticoid receptor

GI-Gastrointestinal

Gp130-Glycoprotein 130

GPx-Glutathione-peroxidaseGREs-Glucocorticoid response elements

HALI- Hyperoxic acute lung-injury

HAP-Hospital-acquired pneumonia

HIF-1-Hypoxia-inducible factor 1

HMGB-1-High-Mobility Group Box-1

Hg-Hemoglobin

HL-60-Human promyelocytic leukemia cells 60

HO-Heme oxygenase

HPA-Hypothalamic-pituitary-adrenal

HR-Heart rate

HS-Hemorrhagic shock

HSP-Heat shock protein

I/R-Ischemic/reperfusion

ICU-Intensive care unit

IFN-Interferon

IκB- Inhibitor of κB

IKK-IKB kinase

IRAK-IL1R-associated kinase

IRF-Interferon regulated factors

iNOS- Inducible nitric oxide synthetase

JAK-Janus kinase

JNK-c-Jun NH_2-terminal kinase

KO-Knock out

LIR-Localized inflammatory response

LPS-Lipopolysaccharide

MAC-Mitochondrial apoptosis-induced channel

MAP-Mean arterial pressure

MAPK-Mitogen-activated protein kinase

MC4-Melanocortin receptor 4

mcl-1-Myeloid cell leukemia-1

MMP-Mitochondrial membrane potential

MOF-Multi-organ failure

MOMP-Mitochondrial outer membrane permeabilization

mPTP- Mitochondrial permeability transition pore

MSH-Melanocyte-stimulating hormone

mtDNA-Mitochondrial DNA

MVR-Mesenteric vascular resistance

MyD88-Myeloid differentiation primary response gene 88

NAD-Nicotinamide adenine dinucleotide

NADH-Nicotinamide adenine dinucleotide hidreto

nGREs-Negative glucocorticoid response elements

NF-κB-Nuclear factor kappa-light-chain-enhancer of activated B cells

NIK-NF-kB inducing kinase

NO-Nitric oxide

NOP-Nociceptin/orphanin FQ peptide

NOS-Nitric oxide synthetase

O_2ER-O_2 extraction ratio

PAMPs-Pathogen associated molecular patterns

PARP-Poly (ADP) ribose polymerase

PBS-Phosphate buffer saline

PCR- Polymerase chain reaction

POMC-Proopiomelanocortin

PPAR γ- Peroxisome proliferator-activated receptor γ

PRR-Pattern recognition receptors

PVN-Paraventricular nucleus

qPCR-Quantitative real-time polymerase chain reaction

RIPK- Receptor-interacting protein kinase

ROS- Reactive oxygen species

RSI-Rapid-sequence-intubation

siRNA-Small interfering RNA

SIRS-Systemic inflammatory iesponse syndrome

SOD-Superoxide dismutase

STAT-Signal transducer and activator of transcription

sTNFR-Soluble TNF receptor

SVR-Systemic vascular resistance

TLR-Toll-like receptor

TNF-α-Tumor necrosis factor α

TRH-Thyrotropin-releasing hormone

TRIF-TIR-domain-containing adapter-inducing interferon-β

TSH-Thyroid–stimulating hormone

T3-Tri-iodothyronine

T4-Thyroxine

TUNEL-Transferase mediated dUTP-digoxigenin nick-end labeling

VO_2-Oxygen Consumption

1. Introduction

Hemorrhagic shock (HS) is still considered a significant cause of morbidity and mortality (Hoyt, 2004). Despite the recent advances observed during the last decades, many aspects of its pathophysiology remain incompletely understood. One remarkable example of this is the recent recognition that a condition which was first described in septic shock also occurs in HS: critical illness-related corticosteroid insufficiency (CIRCI). CIRCI is defined as an inadequate glucocorticoid intracellular anti-inflammatory activity for the severity of the patient's illness (Marik, 2009). CIRCI has been demonstrated in HS in both clinical (Cotton et al., 2008; Hoen et al., 2002) and experimental studies (Rushing et al., 2006; Wang et al., 1999). CIRCI has also been associated to increased patient´s morbidity and mortality in HS, sepsis and trauma(Stein et al., 2013; Marik et al., 2008).

The pathophysiology of HS-associated CIRCI is poorly understood. Proposed mechanisms include the development of adrenal dysfunction due to adrenal necrosis caused by HS-associated ischemia (Rushing et al., 2006; Kajihara, Malliwah, Matsumura, Taguchi & Iijima., 1983), reduced adrenal levels of corticosterone (CS) and cyclic adenosine monophosphate (AMPc), reduced corticotropin-stimulated release of CS and decreased adrenocorticotropic hormone (ACTH) secretion (Wang et al., 1999). Wang et al. (1999) also demonstrated that reduced hepatic 11β-hydroxysteroid dehydrogenase (11β-HSD), the hepatic enzyme which catabolizes CS into its inactive form, 11- dehydrocorticosterone, has decreased activity in rats submitted to HS (Wang et al., 1999). This led the authors to suggest that this decreased activity contributes to persistently high levels of CS, which then decrease ACTH secretion, causing a secondary adrenal dysfunction (Wang et al., 1999). It has also been suggested that CIRCI following HS is related with the inflammatory consequences of this condition (Hoen et al., 2002).

Etomidate is an anesthetic commonly used for rapid sequence intubation (RSI) in HS due to its favorable hemodynamic profile (Forman, 2011). It is also known from its ability to directly inhibit adrenal gland steroidogenesis, including after single bolus administration (Allolio et al., 1985; Wagner, White, Kan, Rosenthal & Feldman, 1984). It is now clear that this adrenal suppression is associated to development of CIRCI in critically ill patients and especially when there is concomitant substrate deficiency (Molenaar et al., 2012). Etomidate´s associated CIRCI has been found in several pathological conditions including sepsis and septic shock (Cherfan et al., 2011; den Brinker et al., 2008; Mohammad et al., 2006), trauma (Cotton et al., 2008;) and burned patients (Mosier, Lasinski & Gamelli, 2014). The significance of etomidate-associated CIRCI has been extensively debated. Some authors are convinced that it is associated to increased risk of side-effects, including increased risk of

death (Sunshine et al., 2013; Chan, Mitchell & Shorr, 2012). By contrary, others statethe opposite, declaring that this adrenal suppression does not put the patient´s safety in risk (McPhee et al., 2013; Baird, Ray, Hay & McKeown, 2010; Jabre et al., 2009). In this regard, the most recently available meta-analysis by Bruder, Ball, Ridi, Pickett & Hohl, (2015) concluded that etomidate administration in critically ill patients is associated with increased risk of developing CIRCI and Multi-Organ Failure (MOF) (Bruder et al., 2015).

If etomidate is associated to CIRCI following HS is still incompletely understood. Two clinical studies about this matter have been published, with opposing results. One clinical study with trauma patients and HS found that etomidate was the only modifiable factor associated to increased risk of development of CIRCI (Cotton et al., 2008). However Hoen et al. (2002), also in trauma patients, did not found evidence of a relation between CIRCI and etomidate administration (Hoen et al., 2002).

Apoptosis is a genetically programmed cell death mechanism recently recognized as one of possible mechanisms that lead to MOF in critically ill patients (Thacker, Robinson, Abel & Tweardy, 2013; Moran et al., 2009; Papathanassoglou, Moynihan & Ackerman et al., 2000; Rollwagen, Yu, LI & Pacheco, 1998). What triggers apoptosis in critical illness is still incompletely understood, although increased levels of cortisol, cytokines, reactive oxygen species (ROS), and other mediators have been suggested as potential triggers (Papathanassoglou, Movnihan, Dafni, Mantzoros & Ackerman, 2003; Papathanassoglou et al., 2000).

Adrenal gland apoptosis has been found in trauma patients (Didenko,Wang, Yang & Hornsby, 1996), in experimental models of sepsis (Liu, Zhang, Han, Lv & Xiong, 2015; Wang et al., 2015; Zhang et al., 2015; Kanczkowski et al., 2013; Flierl et al., 2008) and in acute necrotizing pancreatitis (ANP) (Yu et al., 2016; Yu et al., 2012). To our knowledge only one study described the occurrence of adrenal apoptosis in HS (Rushing & Britt, 2007). In some studies the occurrence of adrenal apoptosis was associated to development of significant adrenal dysfunction (Liu et al., 2016; Yu et al., 2016; Yu et al., 2012; Polito et al., 2010). However to our knowledge, if HS is associated to development of adrenal gland apoptosis and if this causes adrenal dysfunction has not been investigated. Etomidate has also effects in apoptosis, which seem dependent from its dosage, target cell and experimental context. Etomidate induces apoptosis in murine leukemia cells (Wu et al., 2011) and exacerbates myocardial apoptosis following ischemia (Xu, Chen, Luo & Firoj, 2014). In contrast, it does not affect human lymphocyte apoptosis induced by stausporine (Roesslein et al., 2008). More recently, three studies in rats have provided evidence that etomidate is clearly associated to adrenal gland apoptosis, through direct and indirect effects (Liu et al., 2016; Liu et al., 2015; Zhang et al., 2015). To our knowledge the effects of etomidate of apoptosis in adrenal gland cells of animals submitted to HS has not been reported.

1.1. Experimental hypothesis and goals of the study

As previously described, current evidence regarding the occurrence of CIRCI in HS is controversial and incomplete. Biomedical research recurs frequently to experimental animal models to mimic human and animal diseases, in an effort to increase the knowledge of its pathophysiological mechanisms, and hopefully, to improve its diagnosis, treatment and prevention. To our knowledge, no experimental model has been described in the literature that addressed HS-associated CIRCI.

One of the main goals of our study was to create a rat model of HS where CIRCI could be investigated. We also wanted to study in particular, the role of adrenal apoptosis and etomidate administration in CIRCI´s development. Based in what has been described previously, it is reasonable to assume that not only HS is associated to adrenal gland apoptosis, but also that this can be related with CIRCI. Regarding etomidate, although its administration has been associated to CIRCI in several conditions including clinical HS, it has not been described in experimental models of this condition. In addition, the clinical significance of etomidate's adrenal suppression in HS is controversial. So, we wanted to develop a model which evaluated if etomidate could aggravate or even be the cause of CIRCI in HS. If that was the case, as a secondary role, we wanted to study the consequences of etomidate-associated CIRCI at several levels, in particular in the inflammatory and endocrine response to HS and in several metabolic and hemodynamic parameters. In addition, we hypothesized that the use of etomidate could also become a way to induce an “artificial CIRCI” state in HS. From a research standpoint, the use of etomidate with this purpose not only would provide useful information about one the commonest drugs used in the management of critically ill patients, but it would also create the possibility of developing a model of CIRCI in HS. This model could greatly increase our knowledge in HS pathophysiology, and in particular, the role of endogenous glucocorticoids in the compensatory response to that condition.

When we were planning the experimental model we chose buprenorphine, as the analgesic to be used in the peri-operative period. Buprenorphine is one of the most commonly used analgesics in experimental animal research (Hubbell & Muir, 1996). Nevertheless, we could not find any data regarding buprenorphine´s effects in adrenal apoptosis. In addition, although buprenorphine is considered an opioid with limited immune and endocrine effects (Lutfy & Cowan, 2004), this hasn´t been rigorously evaluated in HS. To avoid possible bias in data interpretation related with buprenorphine, we created an additional group of animals (G1), with the purpose of evaluating its effects in the endocrine and immune responses and in adrenal gland apoptosis and necrosis.

Our primary hypothesis was that HS was associated to development of CIRCI and that this was related with the development of adrenal gland apoptosis and potentiated by etomidate administration. As secondary hypotheses we considered the following:

- The development of CIRCI was associated to significant morbidity, namely in immune, metabolic and hemodynamic parameters;
- The use of etomidate potentiated the side-effects of HS-associated CIRCI, especially in immune, metabolic and hemodynamic parameters;
- The use of etomidate modulated the occurrence of adrenal gland apoptosis.

To achieve this we measured several variables. To evaluate the Hypothalamic Pituitary Axis (HPA) axis response we measured ACTH and CS plasma levels. The inflammatory response was evaluated by measuring the plasma levels of tumor necrosis factor- α (TNF-α), interleukin-6 (IL6), and interleukin-10 (IL10) and by determining the circulating levels of messenger ribonucleic acid (mRNA) of the genes *TNF-α*, *IL6*, and *IL10*. Hemodynamic parameters include heart rate (HR) and mean arterial pressure (MAP). Several biochemical parameters were also assessed including blood gas analysis and metabolic indicators of tissue perfusion such as lactate, bicarbonate and base excess (BE). Finally adrenal gland apoptosis and necrosis were assessed in both adrenal glands. Apoptosis was assessed by transferase mediated deoxyuridine triphosphate (dUTP)-digoxigenin nick-end labeling (dUTP-TUNEL) staining and active caspase-3 immunofluorescence analysis. All these variables were evaluated regarding its association to CIRCI.

These procedures will be described in more detail in the Methods section.Before this, a literature review regarding HS will be presented.

2. Literature review.

2.1. Importance of Hemorrhagic Shock

The annual incidence of deaths due to traumatic injury is around 5 million People, which corresponds to 10% of all global deaths (Peden & Hyder, 2002). It has been estimated that 30% of these deaths are due to HS (Hoyt, 2004), making this condition one of the major causes of mortality due to trauma (Sobrino & Shafi, 2013). Besides, the presence of HS is a predictor of poor outcome in the injured patient because it leads to complications including multi-organ failure and aggravates morbidity (Angele, Schneider & Chaudry, 2008). A low flow state in many instances similar to HS can also occur in the context of elective surgery and solid organ transplantation (Jarrar, Wang & Chaudry, 2004). The mortality of this non-traumatic HS seems to be similar to trauma associated HS (Jarrar et al., 2004)

In recent years, major improvements in the care of traumatic patients have occurred, which include improvements in the therapeutic approach to HS, leading to a reduction in early-mortality (Kahl et al., 2013). However, late-mortality associated to HS, including those caused by MOF has not changed significantly (Sauaia et al., 2014). Because treatment of MOF-associated HS is still largely unsuccessful, its prevention remains the best strategy (Sauaia et al., 2014). For this to be achieved, a better prevention and/or correction of early and late abnormalities which are induced by HS must be accomplished. However the knowledge about HS pathophysiology is also incomplete. An example of this is the recent recognition that HS is associated to the state of CIRCI, whose underlying mechanisms and consequences are still being debated.

2.2. Hemorrhagic shock-definition and physiological concepts

Much has been discovered since eminent doctors such as John Collins Warren or Samuel Gross have described shock as “a momentary pause in the act of death” or “a rude unhinging of the machinery of life” respectively (Mcswain, Salomone & Frame 1999). The developments of last decades in the knowledge of the condition led to the current definition of shock as a *disorder that result from an inadequate oxygen delivery to the tissues* (DO_2)*, leading to a state of cellular energetic failure and inadequacy in fulfilling tissue metabolic demands* (Munford 2005, p.1606). The result is cellular dysfunction and death by necrosis or apoptosis contributing to organ dysfunction.

HS constitutes one of several recognized types of shock. In reality represents a particular subtype of hypovolemic shock, which results from the loss of an important amount of blood, leading to decreased intravascular volume and blood´s oxygen carrying capacity (Ganong

2006). HS is also a syndrome that includes the consequences of this blood loss, including the direct pathological consequences of the decreased DO_2 and the body´s compensatory response which aims to restore hemostasis.

It is now known that HS initiates a complex host response characterized by different levels of integration and organization, from the subcellular (molecular) and cellular level, with production and release of several inflammatory mediators to the macrocellular level (Lagoa et al., 2006). The macrocellular response consists in several components, including a local tissue and global response that are coordinated by the host´s immune, endocrine and nervous system. Both the micro and macrocellular responses are highly coordinated and integrated through several mechanisms highly conserved between species and which have been selected by natural evolution.

Before the description of the body´s response to HS, it is useful to review very briefly some physiological concepts, and in particular the definitions of DO_2 and its determinants. This will be followed by a review on how HS initiates injury at a cellular level, than at a local and tissue level and finally the general response to HS in its neuroendocrine and immune components and its consequences.

2.2.1. Physiologic concepts- DO_2 and Oxygen Consumption (VO_2)

Because current definitions of shock place emphasis in the delivery of oxygen to the tissues, a brief physiologic review on how the body´s oxygenate tissues will be discussed.

An adequate tissue oxygenation depends from two main factors: the rate of DO_2 and the rate of VO_2.

Oxygen uptake is the amount of oxygen that diffuses from capillaries to mitochondria. Tissue oxygenation is adequate when tissues receive sufficient oxygen to meet their metabolic needs. This only occurs if oxygen is effectively transported from the external environment to the tissues.

O_2 utilization by cells depends that this is adequately transported from the lung into the interstitium. O_2 transport in turn results from two processes: convection and diffusion.

Oxygen Convection

Convection is a process where O_2 is transported by the circulation (macrocirculation and microcirculation) to the O_2 exchanging microcirculatory system using hemoglobin as a carrier. It is mainly related with DO_2.

DO_2 results from the product of cardiac output (CO) and arterial oxygen concentration (CaO_2) by the following equation:

Equation 1: $DO_2 = CO \times CaO_2 \times 10$.

CO is dependent from the equation:

Equation 2: CO: SV x HR,

SV is stroke volume and HR heart rate. SV will depend from variables such as preload, afterload, heart muscle contractility and rhythm.

Equation 1 may also use cardiac index (CI) instead of CO. CI is CO divided by the body surface area. Equation 1 then becomes equation 3:

Equation 3: $DO_2 = CI \times CaO_2 \times 10$

Normal values for CI in Humans are 2.5 to 4 L/min/m^2 and normal DO2 of 640 to 1400 mL/min or 500 to 600 mL/min/m^2.

It can be concluded by equations 1, 2 and 3 that an adequate DO_2 depends from the normal function of the cardiovascular system and from the adequate content of O_2 in blood.

CaO_2 may be calculated by the following equation:

Equation 4: $CaO_2 = (Hg \times 1.39 \times SaO_2) + (PaO_2 \times 0.003)$

Where Hg is the hemoglobin level, SaO_2 the arterial saturation of hemoglobin by oxygen, PO_2 the arterial partial pressure of O_2, and 0.003 the solubility coefficient of O_2 in human plasma. Each gram of hemoglobin is capable of carrying 1.39 mL of O_2. The amount of oxygen carried on the hemoglobin is Hg x 1.39 x SaO_2.

Free O_2 has a low solubility in plasma. Thus CaO_2 and venous oxygen concentration (CvO_2) are mainly dependent from O_2 carried by Hg in red blood cells. Consequently it is usually acceptable to use the abbreviated equation for CaO_2:

Equation 5: $CaO_2 = Hg \times 1.39 \times SaO_2$.

However, when a high inspiratory fraction of O_2 (FiO_2) occurs, then the amount of dissolved O_2 becomes important. In these cases one should use equation 4.

Once Hg is completely saturated with oxygen (HbO_2), oxygenation can be increased by increasing PO_2. This may be achieved by either increasing its FiO_2 or by increasing the pressure of the air-oxygen mixture into the alveoli (intra-alveolar pressure).

In addition convection may be related to VO_2 by the Ficks law of convection.

Equation 6: $VO_2 = CO \times (CaO_2 - CvO_2) = CO \times CaO2. [(CaO_2 - CvO_2)/ CaO_2]$

CvO_2 is venous oxygen concentration can be obtained by the following equation:

Equation 7: CvO_2= (Hb x 1.39 x SvO2) + (PvO2 x 0.003),

where SvO_2 is the venous saturation and PvO_2 the venous partial pressure of O_2

The normal VO_2 in Humans is 180 to 280 mL/min or 110 to 160 mL/min/m^2

O_2 extraction ratio (O_2ER)

Another important variable is O_2ER. This represents the fraction of DO_2 that diffuses from capillaries to tissues, expressed as percent of total. O_2ER is defined by:

Equation 8: $O_2ER= [(CaO_2-CvO_2)/CaO_2]= VO_2/DO_2$

O_2ER at cellular/tissues level is the main driver, controller and modulator of the DO_2 system. Its normal values in Humans range from 25 to 30%.

Oxygen Diffusion

The diffusion O_2 of from the capillaries to the cells in the interstitium is related with VO_2 by the Fick´s law of diffusion, where

Equation 9:$VO_2= KO_2 x (PcO_2-PmitO_2)$

KO2 is a parameter dependent from the diffusing capacity for O_2 in the microvascular network. It depends from the capillary surface area and from the path length from the capillaries to the mitochondria. PcO2 is the mean capillary pressure of PO_2 and $PmitO_2$ is the PO_2 in the space surrounding the mitochondria (the final destination of O_2).

The greater the pressure gradient and the capillary surface area, the higher will be the number of O_2 molecules to diffuse. The diffusion distance is inversely related to the rate of diffusion.

2.3. HS injury at the molecular /cellular level

2.3.1. *Hypoxia*

Hypoxia is defined as the diminished availability of O_2 into the tissues. Although there are many causes of hypoxia, in general hypoxia can be due to three main causes:

- Decreased O_2 supply by the macrocirculation;
- Maldistribution of O_2 supply;
- Impairment of O_2 consumption.

Hypoxemia in contrast, represents decreased CaO_2. Its main causes are the following:

- Decreased FiO_2;
- Alveolar hypoventilation;
- Impaired diffusion at the alveoli-blood barrier;
- Ventilation perfusion mismatch due to cardiac and pulmonary diseases;
- Shunt (which is an extreme case of a ventilation-perfusion mismatch)

HS can be associated to both hypoxemia and hypoxia. The two terms should not be used interchangeable, although in many aspects its pathophysiologic mechanisms and therapeutic approaches are similar.

2.3.2. *Ischemia*

HS is conceived as a global ischemic injury where a significant part of its morbidity and mortality is attributed to the imbalance between decreased systemic DO_2 caused by decreased tissue perfusion and VO_2. Because O_2 cannot be stored in large amounts, aerobic metabolism is only possible when DO_2 and VO2 are adequately matched (Jarrar et al., 2004). If this does not occur the result is development of cellular hypoxia (Angele et al., 2008).
Cellular hypoxia in HS results mainly from *ischemia* which is defined as “the condition that occurs from the loss of blood supply due to impeded or insufficient arterial blood flow, leading to decreased DO_2 and metabolic substrate availability to cells” (Dogan & Aslan, 2011). It is than ischemia which initiates the cascade of events that surrounds the pathophysiology of HS.
When faced with hypoxia, cells begin to produce adenosine triphosphate (ATP) by glycolysis, which is less productive than the more efficient oxidative phosphorylation. Depending from the amount of ATP generated, the cell may be able to maintain its functions, including protein synthesis and contractile processes. If hypoxia is enough then cellular dysfunction ensues. The latter may become irreversible leading to apoptosis or necrosis depending how severe is the decrease in ATP. The resistance to hypoxia depends from cell type, and in particular the intrinsic metabolic rate and adaptive mechanisms present in the different tissues (Li & Jackson, 2002). For instances hepatocytes can endure 2.5 hours of ischemia without showing evidence of irreversible damage (Schumacker, Chandel & Agusti, 1993).By contrast cells from the gastrointestinal tract (GI) (Dubin et al., 2001) and brain (Erecińska & Silver, 2001) are particular susceptible to ischemia.
The accumulation of the glycolysis end-products, pyruvic and lactic acid lowers the cytosolic and mitochondrial matrix pH. A lower pH combined with decreased ATP levels concurs to the decrease of the sodium /potassium ATPase pump activity, leading to increased Na^+

accumulation inside the cell. This event is followed by water accumulation and cellular swelling, which cause loss of mitochondrial matrix and disintegration, expansion and formation of vesicles in the endoplasmic reticulum (ER) and cytoplasm and lysosomal rupture, with subsequent release of enzymes into the cytoplasm (Belzer & Southard, 1988).
Membrane Ca^{2+} pumps, also driven by ATP, begin to fail with decreased oxygen availability. As a result cytosolic Ca^{2+} concentration increases, which augments the activity of calcium channels existent in the ER. Once these activated, occurs an increase in the Ca^{2+} efflux from that organelle to the cytoplasm. Increased cytosolic Ca^{2+}concentrations is a critical step for cellular injury, because it activates phospholipases, proteases, ATPases and endonucleases (Chaudry, 1983). Once activated these enzymes lead to membrane damage and structural and membrane protein loss. They also induce further ATP depletion and DNA fragmentation.
During Health, O_2 availability is related with the redox state of the cell. In fact one of the hallmarks of HS is the decoupling that occurs between the cell´s redox state and the acceptance of electrons by in the mitochondria (Jarrar et al., 2004). The decoupling that occurs in the mitochondrial electrical transport chain (Li & Jackson, 2002) due to the lack of oxygen can lead to increased production of Reactive Oxygen Species (ROS). The latter may also result from the increased cytosolic Ca^{2+} levels, which increase mitochondrial membrane depolarization and decrease mitochondrial potential. Many types of ROS have been identified which result from these processes. However, in HS, the ones which seem to be more clinically significant are superoxide (O_2^-), hydroxyl (HO^-) and the highly reactive specie peroxynitrite ($ONOO^-$). The latter is formed in the presence of nitric oxide (NO) (Fink, 2002).
ROS-induced injury can occur by three major mechanisms:

- A direct toxic effect of ROS in cellular components inducing lipid peroxidation, oxidation of critical thiols groups in enzymes, and structural protein and nucleic acid damage;
- An indirect effect mediated via activation of cell signaling pathways culminating in the generation of pro-inflammatory mediators;
- Augmenting Toll-Like-Receptors (TLR) crosstalk.

Moreover, ROS overwhelm the endogenous antioxidant mechanisms thereby rendering tissues more susceptible to oxidant damage (Xiang, Fan & Fan, 2010). One mechanism how ROS lead to generation of pro-inflammatory mediators is through the increased activity of the transcription factor Nuclear Factor k-Beta (NF-kB). NF-kB can increase the expression of cytokine genes such as TNF-α, IL1 and IL6.
As described before one the most toxic ROS is peroxynitrite. Peroxynitrite induces cellular damage through several mechanisms including tyrosine nitration, lipid peroxidation, direct and irreversible inhibition of mitochondrial respiratory chain enzymes, inactivation of

glyceraldehyde-3-phosphate dehydrogenase, inhibition of membrane sodium/potassium ATPase activity, inactivation of membrane Na^+ channels, and other oxidative modifications of proteins (Szabo & Módis, 2010). It is also a potent trigger of DNA single strand breakage, with leads to subsequent activation of Poly (ADP) ribose polymerase (PARP-1).

PARP-1 is a nuclear enzyme that performs several cellular functions including the repair of single-strand breaks in nuclear DNA, DNA replication and apoptosis (Fink, 2002). To perform these actions, it catalyzes the cleavage of nicotinamide adenine dinucleotide (NAD^+) into ADP ribose and nicotinamide and the polymerization of the obtained ADP-ribose blocks into branching poly (ADP-ribose) homopolymers. Through its activity PARP-1 depletes NAD/ nicotinamide adenine dinucleotide hidreto (NADH) intracellular contents. In states of acute inflammation, increased production of ROS increases the presence of single-strand breaks in nuclear DNA, thus activating PARP-1. The subsequent decrease in NAD^+/NADH intracellular content can impair oxidative phosphorylation because NADH is the main reducing equivalent that supports that process (Wendel & Heller, 2010). Consequently, this leads to a further impairment in the cell´s ability to produce ATP, in a process which has been called as cytopathic hypoxia (Fink, 2002).

In recent years the importance of mitochondrial dysfunction and cytopathic hypoxia in the development of MOF in sepsis and other critical illnesses has been increasingly recognized (Kozlov et al., 2011). Although in ischemia/hypoxia, mitochondrial damage can result from several mechanisms, including increased levels of intracellular Ca^{2+} and phospholipid breakdown through increased phospholipase A_2 activity, it is currently believed that the major contributor is increased ROS production, in particular of peroxynitrite (Wendel & Heller, 2010). Once mitochondrial damage becomes established, several pathophysiologic consequences occur which may culminate with cell death. Mitochondrial damage is characterized by a decrease in its membrane potential. This causes an increase in the permeability of the mitochondrial membrane, leading to an efflux of mitochondrial pro-apoptotic factors to the cytoplasm, including cytochrome c and apoptosis-inducing factor (AIF) (Kozlov et al., 2011). The latter will initiate the process of apoptosis by activating several intra-signaling cascades (discussed later). In addition mitochondrial proteins and DNA released from apoptotic and necrotic cells function as damage-associated molecular patterns (DAMPs) to activate innate immunity and initiate sterile-inflammation (Hirsiger et al., 2012; Castellheim et al., 2009) (discussed later).

In many cell types submitted to ischemia/hypoxia, the enzyme inducible nitric oxide synthetase (iNOS) is activated through the action of hypoxia-inducible factor 1 (HIF-1) (Li & Jackson, 2002), leading to an increase in NO production. NO can have both protective and deleterious effects in HS (Szabo & Billiar, 1999). Some of its deleterious effects result from

NO reaction with O_2^- to produce the toxic peroxynitrite, a powerful oxidant substance (Szabo & Billiar, 1999).

2.3.3. *Ischemia / Reperfusion injury (I/R)*

Several studies have highlighted that HS-associated injury does not depend solely from ischemia and subsequent cellular hypoxia. In fact, it is now clear that the initial injury caused by ischemia is aggravated when tissue perfusion and reoxygenation is restored, the so-called I/R injury (Angele et al., 2008). Interestingly there is evidence that suggests that in HS, although therapeutic measures which aim to restore an adequate DO_2 are essential to prevent ischemic death, they can also be harmful, because they initiate I/R injury. Studies have also demonstrated that injury due solely to ischemia is milder than the one induced by the combined effects of ischemia and reperfusion (Younes et al., 1984). It was also shown that if reperfusion is achieved in hypoxic conditions, post-ischemic microvascular injury is attenuated when compared with reperfusion accompanied with high levels of oxygen supplementation (Korthuis, Smith & Carden, 1989). This supports the "pathogenic role of oxygen" in reperfusion.

There is abundant evidence that demonstrates that I/R injury is mainly caused by ROS, which are produced when oxygen supply is restored (Eltzschig & Eckle, 2011). The biochemical pathways that lead to the formation of these chemical mediators are now reasonably understood. During the ischemic phase, ATP is degraded into hypoxanthine. In normal conditions, hypoxanthine is degraded by xanthine dehydrogenase (XD) into xanthine. However in ischemic tissues, XD is converted into xanthine oxidase (XO). Contrary to XD, which has NAD as a cofactor for its catalytic activity, XO depends from oxygen. Consequently, during ischemia XO cannot degrade effectively hypoxanthine which than accumulates excessively. Once oxygen supply is restored to the cell, the excessive hypoxanthine is converted in ROS by XO (Collard & Gellman, 2001). Other sources of ROS formation in HS include hemoproteins oxidized to their ferryl form, such as ferrylhemoglobin, NADPH-oxidases and redox cycling of intracellular iron (Li & Jackson, 2002).

2.3.4. *The "danger theory" applied to HS*

In recent years it has been acknowledged that in conditions such as severe trauma and shock, the host´s innate and adaptive immune system can react to endogenous signals released from stressed, injured and necrotic cells in the same way that reacts to exogenous pathogens (Castellheim et al., 2009). Moreover, it has been found that the endogenous signals activate the immune system through the same receptors which are activated by the

exogenous stimulus. These findings challenged the classical accepted view that the immune system worked basically by distinguishing “self” from “non-self”. However they could be easily explained by the “danger “theory which was first introduced in 1994 (Maztinger 1994). Accordingly to this, the immune system recognizes signals as “danger” through its ligation to specific receptors named “pattern recognition receptors” (PRR). Import types of PRR which have been implicated in HS pathophysiology are the TLR and mannose receptors, which are present in monocytes, macrophages, polymorphonuclear neutrophils and dendritic cells (Denk, Perl & Huber-Lang, 2012). One of the TLR which has been proved to be fundamental in the pathophysiology of HS is TLR-4 (McGhan & Jaroszewski, 2012; Midwood, Piccinini & Sacre, 2010).

The signals which were first identified as “danger signals” and which are recognized by PRR were exogenous molecules originated from pathogens. Consequently they have been named as pathogen associated molecular patterns (PAMPs). PAMPs are still believed to play a role in the pathophysiology of HS (Angele et al., 2008). In fact it is believed that in HS, invading pathogens and their PAMPs translocate through a damaged intestinal epithelium affected by I/R injury, and initiate a correspondent inflammatory response. In contrast, the danger signals which are endogenous and are originated from stressed, necrotic and even apoptotic cells were named damaged-associated molecular patterns (DAMPs) or alarmins (Hirsiger et al., 2012).

It is now recognized that several alarmins are important for initiating the host´s immune response to HS. Most alarmins fall into two categories. The first category includes intra-cellular molecules, normally inaccessible to the immune system, which are released after cell´s injury or death through necrosis and/or apoptosis, or by cell activation. The second category compromises molecules or molecular fragments from the extracellular matrix which are released with tissue damage or are specifically upregulated in response to tissue injury (Midwood, Piccinini & Sacre, 2010). Examples of alarmins which have been associated to the inflammatory response of HS include heat–shock proteins (HSP), hyaluronan, uric acid, galectins, thioredoxin, adenosine, High-Mobility Group Box-1 (HMGB-1), interleukin1α (IL1α), interleukin-33 (IL33), ATP and mammalian and mitochondrial DNA (Hirsiger et al., 2012; Kozlov et al., 2011). Interestingly HMGB-1, IL1α and IL33 also function as extracellular mediators and intracellular transcription factors and so they are called dual function alarmins (Hirsiger et al., 2012). The release of DAMPs, ROS, ischemia and I/R combine to initiate a vicious cycle of tissue injury and immune activation (see figure 1).

Figure 1: The distinct stages of tissue I/R injury.

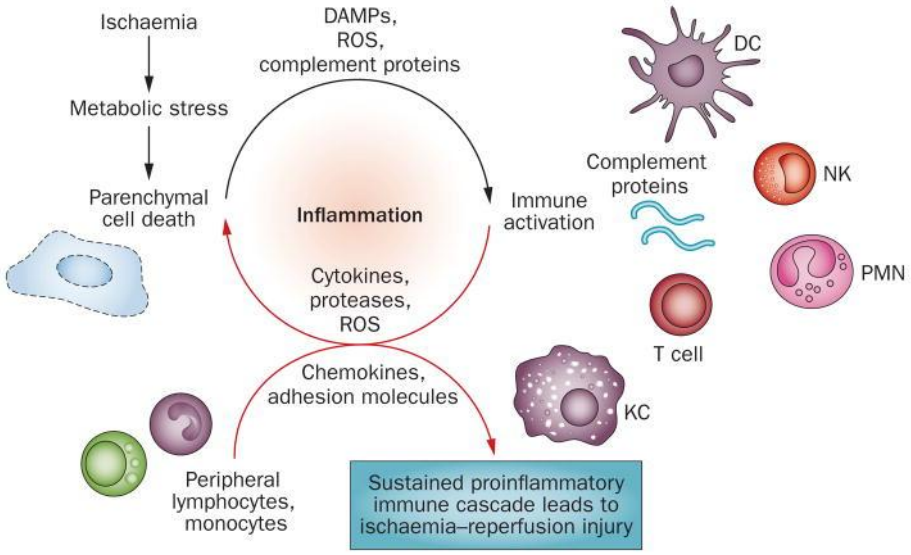

Figure 1: Ischaemic injury, a localized process of tissue metabolic disturbances, results from glycogen consumption, lack of oxygen supply and ATP depletion. The cell-death-released DAMPs, activation of complement (a group of proteins that are involved in tissue injury and/or repair) induced by tissue injury and mitochondrial ROS production triggered by oxygenation all contribute to immune activation after reperfusion, which involves multiple cell types, including macrophages, dendritic cells, T cells, NK cells and neutrophils. The I/R-activated proinflammatory immune cascade sustains itself by recruiting peripheral immune cells from the circulation, and is responsible for the ultimate tissue reperfusion injury. Abbreviations: DAMPs, danger-associated molecular patterns; DC, dendritic cells; NK, natural killer cell; PMN, polymorphonuclear cells; ROS, reactive oxygen species. Adapted from Zhai, Petrowsky, Hong, Busuttil & Kupiec-Weglinski, 2015.

HMGB-1 was originally described as a DNA-binding protein present in all nucleated cells which acts as a transcription factor (Hirsiger et al., 2012). Later it was recognized as a pro-inflammatory cytokine in sepsis and endotoxemia and an endogenous trigger of the inflammatory response in sterile injury such as HS and I/R injury (Tsung et al.¸2005). In these conditions HMGB-1 is mainly released by active secretion by macrophages and monocytic cells. Other cell types can also secrete HMGB-1 once exposed to proinflammatory cytokines, C5a and bacterial products. HMGB-1 may also be passively released by necrotic or disrupted cells or during late apoptosis due to increased cellular permeability and nucleosomal degradation (Harris, Andersson & Pisetsky, 2012). HMGB-1´s pro-inflammatory effects include activation of phagocytic and endothelial cells and loss of epithelial barrier functions. It can also act by binding to endogenous and exogenous inflammatory mediators, such as cytokines or endotoxins and induce the typical signs of

inflammation and other symptoms, collectively referred to as “sickness syndrome”. How HMGB-1 performs these effects is still incompletely understood although they may result from its interaction with PRRs such as TLR2 and TLR4.

The IL1 super-family is composed by 11 members, which are involved in inflammation. There are two types of IL1: IL1α and IL1β. IL1α is a dual function alarmin which induce an inflammatory response (Bertheloot & Latz, 2016). It is also constitutively expressed in epithelial cells, keratinocytes and fibroblasts, which can secret it in an active manner although this is rare. pIL1α, the precursor of IL1α is present in the cell´s cytoplasm and acts as a nuclear transcription factor. When the cell is exposed to inflammatory stimulus, including lipopolysaccharides (LPS) or TNFα, pIL1α is translocated into the nucleus, where it directs the production of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and proinflammatory cytokines, such as IL6 and IL8. In contrast to pIL1α, the biological role of constitutively expressed IL1α is not entirely clear. When there is loss of cellular integrity by necrosis, IL1α reaches the extracellular milieu, where it exerts its functions. Interestingly this does not occur when cell death occurs by apoptosis because in this case IL1α remains attached to chromatin. This reduces its intracellular mobility and possibly limits its passive release. Monocytes and macrophages do not constitutively express IL1α but are capable of synthesizing de novo IL1α once activated. When this occurs, membrane calpain cleaves pIL1α, originating IL1α which is than secreted.

Thus there are two main sources of IL1α: in the majority of tissues IL1α is only released by necrotic cell disintegration; however in monocytes and macrophages, IL1α is derived mainly by active secretion. IL1α inflammatory effects include inducing lymphocyte activation, acting as a costimulant on natural killer (NK) cell activity and enhancement of monocytes and macrophage´s defensive activity through production of several inflammatory mediators (Bertheloot & Latz, 2016).

IL33 is the most recently identified dual function alarmin. It is mainly expressed in structural and lining cells, including endothelial cells, fibroblasticreticular cells of lymphoid tissues, and epithelial cells of tissues exposed to the environment. In the absence of inflammatory stimuli, IL33 is normally located in the cell´s nucleus. Similar to HMGB-1 and pIL1α, IL33 function both as a transcription factor and as a cytokine. IL33 exerts cytokine activity when is passively released from necrotic tissues. However, contrary to IL1α and HMGB1, as a transcription factor IL33 exerts repressive transcriptional activity and as a cytokine has anti-inflammatory properties and promotes the resolution of inflammation (Kunes et al., 2010)

2.4. The Body´s compensatory response to HS at a subcellular level

The combination of hypoxia/ischemia and reperfusion injury originates a compensatory response by the body to restore hemostasis. This response involves different levels of increasing complexity, beginning at intra-cellular level (genome, transcriptome, proteome, physiome) and culminating with a multi-organic response (Cobb & O´Keefe, 2004) (see figure 1 and 2). It is highly coordinated and integrated and in most of the times beneficial. However when it becomes prolonged and/or is too excessive, its effects become more deleterious and may actually contribute to the pathophysiology of HS.

2.4.1. *Response at the genetic level*

As it was discussed, ischemia/hypoxia and I/R injury can directly change cellular activity by affecting several cellular functions. The cell, in an attempt to react and adapt to these insults initiates several protecting mechanisms. These include activation of several intra-signaling pathways, changes in cellular metabolism and secretion and gene expression (figure 3). These mechanisms have several goals including the repair of injured cellular structures, restoration of cellular functions, arrest of the cell cycle and neutralization and/or containment of injury. If they fail and the injury is irreversible the cell dies by apoptosis or necrosis. Some of these mechanisms are ubiquitous and present in all types of cells (e.g. heat–shock response), whereas others are more cell-specific (e.g. the production and secretion of several cytokines). Some are directed to a specific actions (e.g. increasing superoxide dismutase (SOD) to degrade ROS) whereas other lead to more general consequences (e.g. the NF-kB pathway). In addition they may be directed toward the injured cell itself or have the purpose to prevent injury in nearby and distant cells. In the next sections the intracellular response to HS will be characterized, beginning by response at the genetic level.

Genetic changes induced by HS

HS triggers profound changes in gene expression in cells throughout the body (Edmonds et al., 2011). Several processes regulated by gene expression and which are changed by HS include stress, inflammatory, immunoregulatory and metabolic pathways (Xiaojun, Cheng, Yuxing & Zhiqian, 2012; Jian et al., 2008). Many of the changes in gene expression induced by HS are likely to be adaptive. However in some cases, they may result in sustained expression or overexpression of deleterious pathways, or deregulation of protective genetic mechanism which than become maladaptive, contributing to the pathophysiology of HS itself (Edmonds et al., 2011).

Until recently HS-induced genetic changes were incompletely understood. However in recent years, advancements in Molecular Biology begin to provide this information in an accurate manner. In turn, the obtained information revolutionized the way we understand the body´s response to HS and to inflammation in general.

Examples of the advancements that were behind this revolution include the use of new powerful techniques that permit the analysis of hundreds or thousands of genes simultaneously, in a cost-effective manner such as microarray analysis and the development of new mathematical models which were made to accurately interpret the large obtained data ((Xiaojun, Cheng, Yuxing & Zhiqian, 2012; Lagoa et al., 2006). Finally new approaches such as "Systems Biology", which combines experimental discovery with mathematical modeling have been developed and open new ways of understanding the dynamic global organization and function of a biologic system (Vodovotz & Billiar, 2008).

Several experimental studies have now been performed to determine how HS changes genetic expression (Chen et al., 2006; Alam, Stegalkina, Rhee & Koustova, 2002). Similar studies have also been performed in clinical patients with other critical illnesses as well (Vanzant et al., 2014; Xiao et al., 2011). They demonstrated that significant changes in gene expression occur very rapidly (within 90 min) after the induction of injury/hemorrhage and that can even affect more than 80% of all cellular functions and pathways. This has led some investigators to use the term “genomic storm” to describe what occurs in terms of genetic expression following HS and other critical illnesses (Xiao et al., 2011). Interestingly these studies make it clear that these changes in genetic expression and function always follow a common pattern of steps independently from the initial type of injury. This suggests that this global genetic response is an ancient and highly conserved mechanism through evolution.

Although a complete understanding about this “genomic storm” is still incomplete, what has been found so far can be resumed in the following:

- The genetic changes are robust and occur very early after injury;
- They occur regardless of the severity of injury (for instances they were also observed following to minor trauma);
- There are unique patterns of gene expression depending from the severity of injury. Genes that are most upregulated with severe injuries include the genes of IL10, IL6, glucocorticoid receptor (GCR) signaling, amino acid metabolism, acute phase response and cell death;
- They are accompanied by epigenetic changes, namely by a decrease in the acetylation of nuclear and cytoplasmic proteins (Li & Alam, 2012);
- There is an upregulation of genes associated with the endoplasmatic reticulum stress response, GCR signaling, acute phase response, death receptor signaling, apoptosis pathways, amino acid metabolism, and the cytokines IL10 and IL6;

- There is rapid downregulation of genes associated to biosynthetic and metabolic pathways and genes associated with antigen presentation;
- The time following injury/resuscitation also affects genetic expression:
    - Genes which are upregulated early after injury include genes associated to GCR signaling, p53 signaling, and IL10 signaling,
    - This is followed by the upregulation of the genes that codify the acute phase response and other inflammatory pathways,
    - The last genes to be upregulated include genes associated to cell death and apoptosis pathways,
- Genes associated to proinflammatory and anti-inflammatory mediators are translated simultaneously.
- Genetic expression is time-dependent. For instances genes involved in the stress response and immunoregulation remained constantly upregulated throughout the course of injury. In contrast, genes involved in cell death and inflammatory pathways are more upregulated later in disease course. The opposite occurs with genes associated with metabolic function, which as the disease progresses, become increasingly down-regulated.
- Genetic expression changes are organ specific. The largest number of changes in gene expression occurs in the liver, followed by lung, muscle and spleen (Chen et al., 2006; Alam et al., 2002). Genetic changes are also very significant in the leukocyte transcriptome (Xiao et al., 2011)
- The type of resuscitation, and in particular, the type of fluid which is used in resuscitation affects genetic expression in an organ-specific manner.
- The type of fluid more associated to changes in genetic expression was found to be plasma, followed by Lactate Ringer and Hypertonic saline (Alam et al., 2002).The organ most affected by hypertonic saline and hetastarch was the lung (Chen et al., 2006)
- The changes in genetic expression that accompanied the development of secondary complications such as nosocomial infection or MOF were not qualitatively different from genetic expression caused by initial injury. They only differed in magnitude (which was higher) and duration (which was prolonged).

So what does this tell us? First that the use of gene array technology and mathematical modeling can provide rapid and efficient means of dissecting the complex genetic response

to shock. Second and contrarily to the previously accepted mechanistic view, at least at a genetic level, severe injury is not associated to an early proinflammatory stage, followed by a compensatory anti-inflammatory state. In contrast it is associated to the simultaneous *increased expression of genes involved in systemic inflammatory, innate immunity, and compensatory anti-inflammatory responses*. In addition it is associated to the suppression of genes involved in adaptive immunity. This is truly a new paradigm that changes completely what it was commonly assumed as the normal body´s reaction to severe injury (figure 1). Besides, the observation that the different types of injuries induce changes in genomic expression which are very similar suggests that the early response to severe inflammatory stress is very ancient and highly conserved by evolution.Finally the fact that it can be changed by resuscitation demonstrates that this response can be modulated. One could envisage therapeutic interventions that can be tailored to modulate this response, especially if it becomes maldaptative. There are experimental studies that have already showed that this approach is feasible. For instances, the use of histone deacetylase inhibitors has been shown to prevent cell death, decrease inflammation, attenuate the activation of pro-apoptotic pathways and promotion of pro-survival pathways (Li & Alam, 2012).

Figure 2: the genomic storm: refining the immune, inflammatory paradigm in trauma

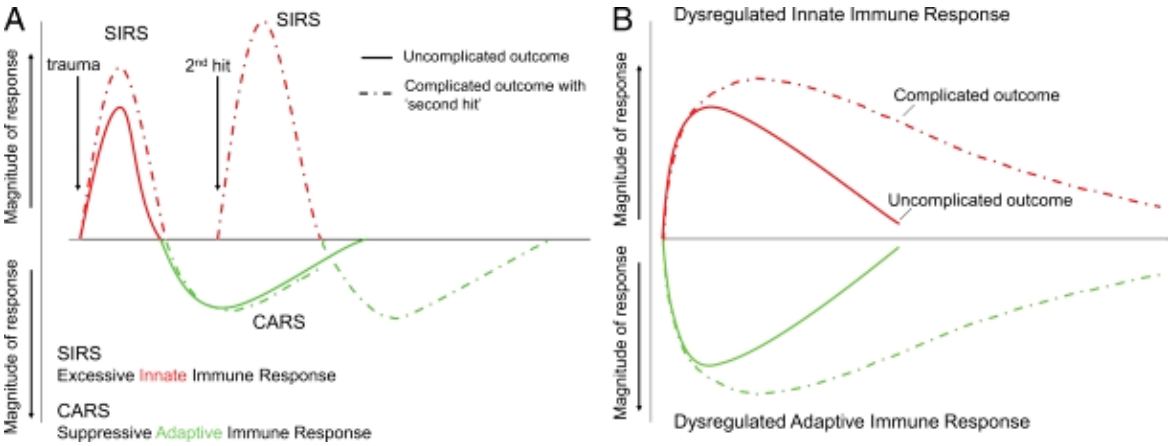

Figure 2: (A) The current paradigm explains complications of severe injury as a result of excessive proinflammatory responses (SIRS) followed temporally by compensatory anti-inflammatory responses (CARS) and suppression of adaptive immunity. A second-hit phenomenon results from sequential insults, which leads to more severe, recurrent SIRS and organ dysfunction. **(B)** The proposed new paradigm involves simultaneous and rapid induction of innate (both pro- and anti-inflammatory genes) and suppression of adaptive immunity genes. Complicated recoveries are delayed, resulting in a prolonged, dysregulated immune–inflammatory state. Adapted from Xiao et al., 2011.

2.4.2. Intra-signaling molecular pathways induced by HS

Besides changes in genomic expression, HS also triggers changes in several intracellular signaling pathways. Most of these are interconnected through a complicated signaling network or web. Depending on how this interconnection is affected may lead to different outcomes. Some components of these intra-signaling pathways are ubiquitous in most cell types and are activated in most type of injuries. One example of this is the NF-kB intra-signaling pathway. Others are more cell- and context specific. Although our knowledge in this field is still progressing, the current thinking is that these intra-signaling pathways are activated to help the host in counteracting or adapting to injury. However in some circumstances they become maldaptative and actually can aggravate it.

The intra-signaling pathways can be activated by both intra and extracellular stimulus. Known intracellular activators of these intra-signaling pathways include ROS, changes in intracellular Ca^{2+} levels and organelle dysfunction (especially ER). These stimuli can directly activate some intra–signaling pathways such as the NF-kB and Activator Protein-1 (AP-1) intra-signaling pathways. In other cases, the intra–signaling pathways area activated indirectly. For example ROS can also activate the NF-kB intra-signaling pathways by activating mitogen activated protein kinases (MAPK) including extracellular signal-regulated kinase (ERK 1/2) and c-Jun NH_2-terminal kinase (JNK) and inhibitor of κB kinases (IKK) (Fan, Ye & Malik, 2001). The MAPK family of proteins plays an important role in apoptosis (Kim & Choi, 2010) and cytotoxicity (Lee et al., 2012), in particular JNK, ERK1 and ERK2 and m38 MAPK (Lee et al., 2012).

Important extra-cellular activators include cytokines, ROS, hormones, DAMPs and PAMPs. As we saw previously, the ligation of DAMPs and PAMPs to TLRs is important in HS pathophysiology. In this regard TLRs ligation seems that it activates two main intra-signaling pathways: the Myeloid differentiation primary response gene 88 (MyD88)-dependent and the TIR-domain-containing adapter-inducing interferon-β (TRIF) pathways (Midwood, Piccinini & Sacre, 2010). The MyD88-dependent pathway is activated by all TLRs except TLR3 and involves the IL1R-associated kinases (IRAK), IRAK-1 and IRAK-4, TNF receptor-associated factor 6, and MAPK. The MyD88-dependent pathway culminates with the activation of the transcription factor NFκB via the IkB kinase complex. The TRIF pathway is activated by TLR3 and TLR4, activates the interferon regulated factors (IRF) family of transcription factors via recruitment of TRIF and results in the synthesis of interferon (IFN).

Major intra-signaling pathways activated in HS

NF-kB is a family of seven structurally related transcription factors which modulates gene expression in various situations which require a rapid and sensitive immune and inflammatory response (Altavilla et al., 2001). NF-kB is activated by cytokines, ROS, bacterial cell wall products, vasopressors, viral infection, and DNA damage (Brasier, 2006). Each transcription factor is a cytoplasmatic protein consisted by two subunits, p65 and p50, which in the quiescent state are associated to Inhibitor of κB (IκB) proteins. The association with IκB proteins prevents the translocation of that subunits to the nucleus. The canonical pathway of NF-kB activation is initiated when IκBα is phosphorylated by IKB kinase (IKK) with its subsequent phosphorylation-induced proteolysis. This will free p65 and p50 subunits, which are now able to translocate to the nucleus (Brasier, 2006). Once in the nucleus, they ligate to the DNA in their DNA binding site and orchestrate the expression of numerous genes.

By contrast, the noncanonical NF-kB activation pathway involves activating the NF-kB inducing kinase (NIK) to stimulate IKKα-induced phosphorylation and proteolytic processing of the 100-kDa cytoplasmic NF-kB2 precursor. Activated NF-kB2 then forms a complex with Rel B and NIK to translocate into the nucleus thereby activating a distinct set of genes (Brasier, 2006).

Genes which are upregulated by NF-kB including the genes of enzymes such as cyclooxygenase 2 (COX-2), lipoxygenase, and inducible iNOS, of cytokines such as TNF-α, IL1, IL6, IL8, and of chemokines, adhesion molecules, cell cycle regulators and angiogenic factors (Brasier, 2006). Interestingly because the actions of the kB subunits are regulated in a cell-type and stimulus-specific manner, this means that the activation of the NF-kB pathway has different effects in different tissues and with different circumstances.

There is ample evidence that increased NF-kB is involved in virtually every form of inflammation, and in diseases which have inflammation or apoptosis as a component (Liu & Malik, 2006). Increased NF-kB activity has been documented in I/R injury (Jones, Brown, Wilhide, He & Ren, 2005), and in HS-induced acute lung injury (ALI), acute respiratory distress syndrome (ARDS) (Sheng et al., 2012) and MOF (Yin et al., 2003). The pathological consequences of increased NF-kB activity have been particularly documented in sepsis. In effect mice which are deficient in NF-kB-dependent genes are resistant to development of septic shock and to septic lethality. In addition the blockade of the NF-kB pathway corrects or ameliorates septic abnormalities, including the development of systemic hypotension, septic myocardial dysfunction, vascular derangement, intravascular coagulation, increased proinflammatory gene expression, tissue neutrophil influx, and microvascular endothelial leakage (Liu & Malik, 2006).

AP-1 is a collective term referring to the dimeric transcription factors composed of Jun, Fos or activating transcription factor (ATF) subunits that bind to a common DNA site, the AP-1-binding site. Once activated AP-1 also regulates the synthesis of a multitude of proinflammatory proteins (Karim, Liu & Zandi, 1997). Activation of the AP-1 pathway has been documented in HS (Paxian, Bauer, Kaplan, Bauer & Rensing, 2002). This pathway seems to be particularly important in the reaction to oxidative stress because it induces the upregulation of the protective gene HO-1 (Paxian et al., 2002; Rensing et al., 2001).
Another important intrasignaling pathway that is activated with HS is the redox-sensitive transcription factor HIF-1 (Zhang et al., 2008; Hellwig-Bürgel, Stiehl, Wagner, Metzen & Jelkmann et al., 2005; Jarrar et al., 2004). HIF -1 is a dimeric transcriptional complex that has been recognized primarily for its role in the maintenance of oxygen and energy homoeostasis. The HIF-1alpha subunit is O_2 labile and is degraded by the proteasome system, following its prolyl-hydroxylation and ubiquitination in normoxic cells.

It is now known that HIF-1 is involved in more roles than oxygen homeostasis. Immunomodulatory peptides, including IL1 and TNF-α stimulate HIF-1 dependent gene expression even in normoxic cells. Both the hypoxic and the cytokine-induced activation of HIF-1 involve the phosphatidylinositol-3-kinase and the MAPK signaling pathways. In addition HSP and other cofactors interact with HIF-1 subunits. HIF-1 increases the transcription of several genes for proteins that promote blood flow and inflammation, including vascular endothelial growth factor, heme oxygenase-1 (HO-1), endothelial NOS and iNOS and COX-2. The pharmacologic activation of the HIF-1 complex can be desirable in ischemic and inflammatory disorders. Although its activation is commonly regarded as beneficial there are also studies in HS which have shown the opposite (Jiang, Huang, Xu, Hu & Li, 2012; Feinman et al., 2010).

Other intra-signaling pathways which have been shown to be activated in HS, by increasing the expression of pro-inflammatory cytokines include the IRF-3/MyD88 independent pathway (Cotroneo, Nemzek-Hamlin, Bayliss & Su, 2012) and the MAPK pathway (Kochanek et al., 2012).

Consequences of intra-signaling pathways activation: beneficial or deleterious

All these pathways converge to increase the synthesis of a large number of proteins involved in the inflammatory and immune responses. These include cytokines (IL1, IL6, TNFα, IL12, IFNs), chemokines, adhesion molecules, co-stimulatory molecules, growth factors, tissue-degrading enzymes (e.g. metalloproteinases), and enzymes that generate inflammatory mediators such as COX-2 and iNOS.

Several lines of evidence have demonstrated that the activation of these intra-signaling pathways contributes to homeostasis and have a physiological purpose. For example NF- kB activity is known to be anti-apoptotic in normal cells and in cells affected by I/R (Jones et al., 2005). NF-kB is also required for hematopoiesis, differentiation and maturation of both myeloid and lymphoid immune cells, adaptive and innate immunity and host defense against invaded bacterial pathogens (Liu & Malik, 2006). It can also play a role in inflammation resolution (Lawrence, Gilroy, Colville-Nash & Willoughby, 2001).

Furthermore, in HS, the activation of these pathways has been shown to be beneficial in some circumstances. For instances experimental studies have shown that when animals are subject to sublethal hemorrhage, they show improved tolerance to subsequent and more severe episodes. It was found that these beneficial effects are associated to increased activity of the NF-kB, of p38 MAPK and of p44/42 and Stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) pathways (Carter, Liu, Fier & Mendez, 2002; Mendez et al., 2000; Kramer et al., 1999). One the other way round, there is evidence that the blockage of these pathways is associated with beneficial effects (Liu & Malik, 2006).

Does it may be asked if the activation of these intra-signaling pathways is protective or deleterious? Although our knowledge about this subject is still incomplete, some considerations can be advanced. The answer is that the activation can lead to beneficial or deleterious consequences depending from the circumstances. Examples of these include type of injury, organ/tissue/cell where the intra-signaling pathway is activated, interaction with other pathways, magnitude and duration of activation and type of stimulus. An example of the latter is the study by Ye, Ding, Zhou, Chen & Liu, (2008). In this study, increased endothelial NF-kB activity was found to be essential in the development of systemic manifestations to sepsis such as hypotension and MOF (Ye et al., 2008). Nevertheless and unexpectedly it did not contribute to eradicate invading pathogenic bacteria (Ye et al., 2008).

Protective intra-signaling pathways

As discussed the previous intra-signaling pathways can be protective or deleterious depending from the circumstances. The next which will be discussed have been shown unequivocally to be associated with beneficial effects in HS. They most likely represent different aspects of a general and ancient cellular defense system against stressors which were conserved through evolution.

Peroxisome proliferator-activated receptor-γ (PPAR- γ)

PPAR-γ is a member of the nuclear receptor superfamily and a ligand-activated transcription factor that regulates the expression of genes involved in lipid metabolism, cell proliferation and inflammatory response. PPAR-γ forms a heterodimer with the retinoid X receptor and upon ligand-activation, binds to the PPAR-response element in the promoter region of genes, which permits genetic transcription. PPAR-γ been demonstrated to exert anti-inflammatory properties in models of sepsis and septic shock, myocardial ischemia-reperfusion, and HS (Zingarelli & Cook, 2005).

Heat shock proteins (HSPs)

The HSP´s cellular response is a very ancient cellular defense system to stress and was identified in innumerous species, from bacteria to Humans. Its main action is to antagonize protein misfolding and/or unfolding during cell stress (Chi & Karliner, 2004). The protective action of HSP can be observed at multiple levels. When cells are submitted to different types of stressors, heat-shock transcription factors become phosphorylated and form trimmers, which are then transported into the nucleus. In there, they bind the heat shock elements within the promoter enhancer regions of HSP. Depending from the specific type of HSP, they can protect against apoptosis, enhance mitochondrial function, repair ion channels, stabilize redox state, contribute to NO-induced protection, and decrease the production of pro-inflammatory cytokines (Chi & Karliner, 2004), such as TNF-α (Meng & Harken, 2002).

HO-1

Hemo-oxygenases are enzymes which catalyzes the rate-limiting step of heme degradation into iron, carbon monoxide and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase (Deshane, Wright & Agarwal, 2005). Three isoforms of HO have been recognized: two constitutively expressed isoforms, HO-2 and HO-3, and one inducible isoform, HO-1. The latter is upregulated in response to several injurious stimuli including the presence of free heme, hyperoxia, hypoxia, endotoxin, heavy metals, oxidative or nitrosative stress, cytokines and other mediators produced during inflammatory processes and by NO (Alcaraz, Fernández & Guillén, 2003). HO-1 has been reported to possess antioxidant, anti-inflammatory and cytoprotective functions (Deshane, Wright & Agarwal, 2005) and probably represents one of the oldest cellular defense systems. Effects attributed to HO-1 activation include inhibition of oxidative damage and apoptosis with subsequent reduction in edema,

leukocyte adhesion and migration and inflammatory cytokine production. It can also control the increased production of NO which occurs in inflammatory conditions.
These benefits can result from direct effects of HO-1 itself, in the modulation of signal transduction pathways or through regulatory interactions with other enzymes, such as COX or due to the end products from its activity such as CO.
The induction of HO-1 has been implicated in numerous clinically relevant disease states including transplant rejection, hypertension, atherosclerosis, lung injury and endotoxic shock (Bach, 2002). It has also been shown to be protective, especially in conditions associated to oxidative tissue injury (Alcaraz, Fernández & Guillén, 2003). Several studies have now been performed that demonstrate that HO-1 induction is associated to beneficial effects in HS (Arimori et al., 2010; Inuoe et al., 2008).

2.4.3. ER stress response, autophagy and apoptosis

The ER stress response and autophagy

The ER is a subcellular organelle which is responsible for the facilitation of protein folding and assembly and involved in several other physiological activities (Khan, Yang & Wang, 2015). Protein folding is crucial for cell survival. In fact normal cells have mechanisms that assure that proteins are correctly folded during its synthesis and properly maintained thereafter. These mechanisms include protein quality control systems which comprise molecular chaperones and intracellular proteases in the cytosol, ER and in the mitochondria (Gregersen & Bross, 2010).It has been shown that under stress and inflammatory conditions, ER may lose homeostasis in its function, leading to misfolding or unfolding of proteins. This process is called ER stress (Khan et al., 2015) and if persistent can lead to cellular dysfunction and eventually death (Kincaid & Cooper, 2007). To prevent it, an ancient and evolutionarily conserved cellular defense system, named the ER response is activated (Sano & Reed, 2013). Basically it consists in an adaptive mechanism, the unfolded protein response, whose aim is to clear unfolded proteins and restore ER homeostasis. The unfolded protein response is performed by the activation of two protein degradation pathways, the ubiquitin-proteasome via ER-assisted degradation pathway and the lysosome-mediated protein degradation via autophagy. The ER-assisted degradation pathway involves the retro-translocation of unfolded ER proteins to the cytosol where they are ubiquitinated and degraded by a system called proteasome. If the amount of misfolded or unfolded proteins exceeds the capacity of the ubiquitin-proteasome pathway, the autophagy pathway is activated as a secondary defense mechanism (Sano & Reed, 2013). (Figure 3).

Figure 3: diagram of ER response

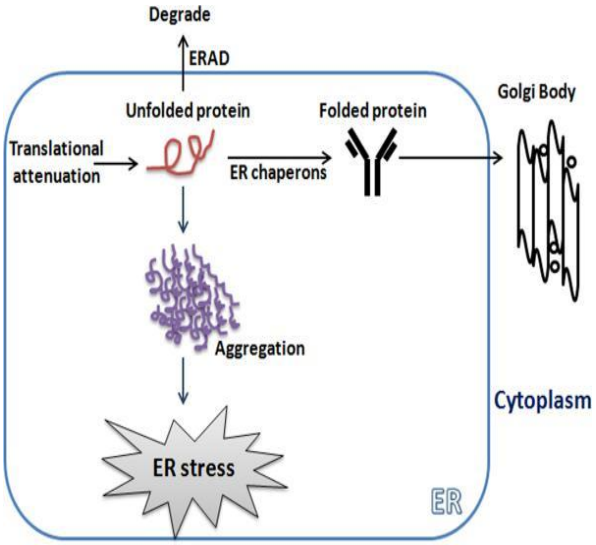

Figure 3: An accumulation of unfolded proteins in the ER induces ER stress. ER stress responses start with: (1) translational attenuation to reduce the further influx of nascent protein; (2) expression of ER chaperones to translocation the folded protein to the Golgi complex; (3) enhanced ER-assisted degradation pathway for degradation of unfolded or misfolded protein; and if unresolved (4) apoptosis. These responses are regulated by the ER localized protein sensors. Adapted from Khan, Yang & Wang, 2015

The presence of ER stress in HS has been described in several studies (Kozolv et al., 2010; Duvigneau et al., 2010; Jian et al., 2008). Potential triggers of ER stress in this condition include hypoxia, oxidative stress, I/R injury and proinflammatory cytokines (Khan et al., 2015; Duvigneau et al., 2010; Kaufman, 2002). If ER stress plays a role in development of apoptosis and organ dysfunction in HS is still being debated.

Autophagy

Autophagy is the process where cellular macromolecules and organellar components are sequestered into vesicles and from here, are delivered to lysosomes for degradation and recycling of bioenergetic substrates (Kaushal, 2012). In this way autophagy is a protective mechanism that degrades potentially toxic products and recycle them for future use. The term autophagy comprises three different processes namely macroautophagy, microautophagy, and chaperone-mediated autophagy (Choi, Ryter & Levine, 2013). Macroautophagy is characterized by the formation of autophagosomes, which encircle isolated cellular components, selectively targeted toxic protein aggregates or impaired organelles such as mitochondria and carry them to lysosomes to form autolysosomes, where they are subsequently degraded by lysosomal hydrolases (Hamacher-Brady, Brady & Gottlieb, 2006).

The current understanding regards autophagy mainly as a protective mechanism by promoting the recycling of new cell components (Kaushal, 2012). However in specific circumstances, especially when there is continuous stimulation by starvation, hypoxia, and inflammation, autophagy can result in cell death due to the overwhelming accumulation of autophagosomes (Dong et al., 2010). Actually autophagy is involved in caspase-independent programmed cell death or programmed cell death type II. To some authors autophagy is actually considered a form of cell death (Shimizu, Yoshida, Tsujioka, Arakawa, 2014).

Autophagy and apoptosis share many regulatory intra-signaling pathways and components of the apoptotic death pathway, including beta-cell lymphoma (Bcl)-2 and beta cell lymphoma extra-large (Bcl-x_L) (Shimizu et al., 2004). The similarity between autophagy and apoptosis and its interconnection actually led some authors to suggest that they represent two events that belong to the same continuum.

In this line of reasoning, following the initial insult, the affected cell can face several outcomes. If the initial injury is not severe and/or the cell has time to respond, the several intracellular defense mechanisms which were discussed previously are activated, in order to minimize or correct resulting abnormalities and to prevent further lesions. One of them is by selective elimination of damaged and pro-apoptotic mitochondria through autophagy.

However if the mechanisms are insufficient to restore cellular homeostasis, moribund cell opts for programmed cell-death which can occur by apoptosis, autophagy or necroptosis (discussed later). The decision to go for apoptosis seems to be related with an insufficient capacity to produce ATP and to maintain Ca^{2+} homeostasis due to lack of functioning and viable mitochondria (Hamacher-Brady, Brady & Gottlieb, 2006). Thus, if the insult is severe to damage innumerous mitochondria and the capacity to produce ATP by oxidative phosphorylation is irreversibly lost, the cell progresses irreversibly to apoptosis.

Autophagy has been described in HS in several studies (Wen et al., 2014; Shen et al., 2013).

Apoptosis in HS

Apoptosis is a genetically programmed mechanism of cell death which is involved in the regulation of many organ functions including embryo development and inflammation (Hattori et al., 2010). It is characterized by several morphological features, including cell and organelle shrinkage, plasma membrane remodeling with blebbing, chromatin condensation and degradation and nuclear fragmentation (Galluzzi et al., 2012). Apoptosis is characterized by the formation of apoptotic bodies which are phagocytized by phagocytic cells, or less commonly by neighbor cells, in a process which does not attract neutrophils and lymphocytes (Majno & Joris, 1995).

Apoptosis can be induced by extrinsic and intrinsic pathways, being called extrinsic and intrinsic apoptosis, respectively. In extrinsic apoptosis, cell death is induced by extracellular signs which are sensed and transmitted through specific transmembrane receptors. Examples of these signs or death ligands include TNF-α, FAS/D95 ligand and TNF-related apoptosis ligand also known as TRAIL (Galluzzi et al., 2012). Independently of the identity of the death ligand, their ligation to the death receptors activates caspases 8, 9 or 10 which than activate the final effector caspases 3, 6 and 7, which through proteolytic activity, induce apoptosis (Galluzzi et al., 2012). In contrast, intrinsic apoptosis results from several intracellular conditions, including intracellular Ca^{2+} overload, DNA damage, oxidative stress and many others. The common final pathway and the point of non-return in intrinsic apoptosis is the mitochondrial outer membrane permeabilization (MOMP). The permeabilization of the outer membrane can occur through activation of two distinct channels: the mitochondrial permeability transition pore (mPTP) in the inner membrane and the mitochondrial apoptosis-induced channel in the outer membrane (Kinnally & Antonsson, 2007). The opening of the mPTP is sufficient to induce apoptosis by itself, through the following mechanisms: it results in a rapid exchange of solutes up to 1.5 kDa in size with redistribution of NADH to the cytosol and an influx of cytosolic water into the mitochondria, causing its matrix expansion. In extreme cases, matrix swelling is sufficient to unwrinkle the inner membrane; however, the outer membrane is less distensible and ruptures, releasing pro-apoptotic factors from the intermembrane space (Gottlieb, 2011).

Once MOMP occurs, this leads to the passage of toxic substances from the mitochondria to the cytosol, such as cytochrome c and other proapoptotic factors. These proapoptotic factors, through several mechanisms activate caspase activity (normally caspase 9). This process is called caspase dependent-intrinsic apoptosis because intrinsic apoptosis can also occur through caspase-independent mechanisms (Galluzzi et al., 2012).

At any moment several proapoptotic and antiapoptotic substances coexist inside cells. It is the balance that occurs between both substances that determine which will be the cell´s fate: to enter in apoptosis or to survive.

Increased apoptosis has been described in sepsis, traumatic brain injury and HS (Cotogni et al., 2010; Bayir & Kagan, 2008) and it is believed to contribute to the pathophysiology of these conditions, in particular to the development of MOF. Apoptosis has been particularly studied in sepsis and septic shock. Lymphocyte apoptosis is currently considered to be one of the major causes of the immunosuppressive state that characterizes these conditions (Hsieh, Athar & Chaudry, 2009). Delayed neutrophil apoptosis was also identified in septic patients and it is believed that it is one of the causes for the extended survival and accumulation of neutrophils in the different tissues (Hsieh, Athar & Chaudhry, 2009). Recently, endothelial cell apoptosis was also associated to increased permeability in HS

(Childs, Tharakan, Hunter, Tinsley, Cao, 2007) and it is considered as a mechanism underlying the systemic capillary leak syndrome (Assaly et al., 2001). The induction of cell apoptosis has also been considered one of the reasons why mitochondrial dysfunction has been associated to MOF in critically ill patients (Kozlov et al., 2011).

Several studies using experimental models of trauma and hemorrhage demonstrated that apoptosis occurs in several organs, including the liver (Yang et al., 2011), small intestine (Lu et al., 2008), kidney (Cotogni et al., 2010), endothelial cells (Childs et al., 2007), splenocytes (Hostmann et al., 2008), thymus (Guan, Jin & Jin, 1998) and lung epithelial cells (Barlos et al., 2008). In these studies apoptosis was not only a consequence of the initial tissue injury but a significant player for the subsequent development of organ dysfunction.

In HS there are many circumstances that push the intracellular equilibrium between pro and anti-apoptotic factors to the proapoptotic side. Not only several death ligands exist in enormous quantities (e.g. TNF-α), which once associated to their receptors will shift the balance to the proapoptotic phenotype, but also several types of cellular changes that lead to intrinsic apoptosis are present (ROS, MOMP, Ca^{2+} overload, ATP depletion, decrease in intracellular pH).

Necrosis and necroptosis

If the lesion is severe enough and/or the cell does not have time to initiate countermeasures the most likely outcome is cellular necrosis. Classical necrosis constitutes a type of non-programmed cell death that occurs after an extreme injury and it is accompanied by organelle swelling and membrane breakdown. Necrosis is also characterized by the activation of inflammation.

In recent years it has been recognized that there are also forms of necrosis which are actively induced by the cell, with specific and defined intra-signaling pathways. In this regard, an active form of necrosis has been recognized and termed necroptosis (Dondelinger et al., 2016). Necroptosis can be triggered by cytokines such as TNF-α, PAMPs and DAMPs (through TLR-3, -4 and -9), I/R injury, Ca^{2+} overload, hypoxia, infectious agents, DNA damage and oxidative and nitrosative stress (Vanlangenakker et al., 2012; Degterev et al., 2005). The most distinctive biochemical marker of this type of cell death is its dependency on receptor-interacting protein kinase 3 (RIPK3) kinase activity (Vanlangenakker et al., 2012). Necroptosis has been recognized in experimental murine models of sepsis (Sharma, Matsuo, Yang, Wang & Wang, 2014; Duprez et al., 2011), traumatic brain injury (Liu et al., 2016), intra-cerebral hemorrhage (Chang et al., 2014) and non-alcoholic steatohepatitis (Afonso et al., 2015).

2.5. The response to HS at the cellular and tissue level

As discussed above, HS leads to widespread cell damage and death, by the combined effects of hypoxia/ischemia and I/R injury. The death of these cells will lead to release of DAMPs to the extracellular environment which are recognized by immune cells, in particular tissue resident macrophages and monocytes, but also by neutrophils and dendritic cells (Kozlov et al., 2011). Through this way DAMP´s and possibly some PAMP´s (see above) induce the activation of cells from the innate immune system almost immediately after initial injury (Figure 4). Once these become activated, various intracellular signaling pathways are initiated in the resident immune cells culminating with the production and secretion of various proinflammatory cytokines, chemokines, and other inflammatory mediators. Examples of these include TNF- α, IL1α, IL6 and IL8, lipid mediators, ROS and tissue-damaging enzymes such as metalloproteinases. Tissue injury and immune cell activation also induce the activation of serine protease cascades such as the coagulation and complement cascades (Rittirsch, Flierl & Ward, 2008). In reality, the extensive cross-talk which occurs between the complement and TLRs present in immune cell´s surface (Figure 5) and molecules from complement, coagulation, fibrinolytic and kallikrein/kinin systems contribute to the almost simultaneous activation of those systems (Brøchner & Toft, 2009; Casthellheim, Brekke, Espevik, Harboe & Mollnes, 2009).

Figure 4: **The inflammatory network in HS**

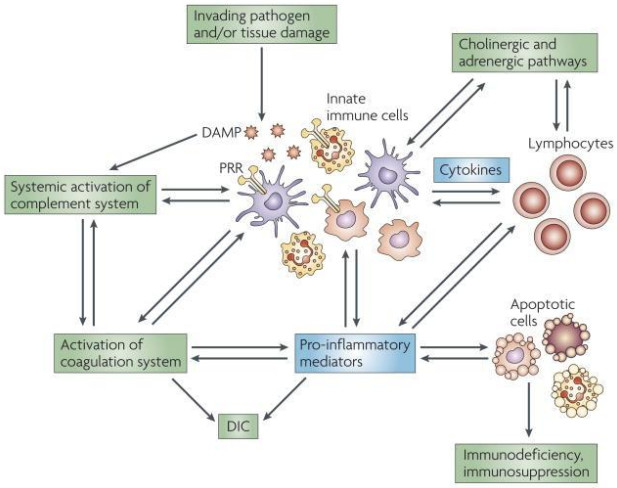

Figure 4: During HS, homeostasis between the various biological systems of the inflammatory network is highly imbalanced. In the initiation of HS, the release of a large amount of DAMPs from invading microorganisms and/or damaged host tissue results in the overstimulation of PRRs on immune cells. Activated immune cells release excessive amounts of pro-inflammatory mediators (resulting in a 'cytokine storm'), free radicals and enzymes, which converts the normally beneficial effects of inflammation into an excessive response that damages the host. Activation of the adrenergic branch of the autonomic nervous system and/or decreased activity of the cholinergic anti-inflammatory pathway (of the parasympathetic branch of the autonomic nervous system) further amplifies the pro-inflammatory responses of neutrophils, macrophages and dendritic cells in sepsis. The presence of invading microorganisms or their products in the blood can cause systemic activation of the complement system, which results in the excessive generation of complement anaphylatoxins, which, at high concentrations, induce innumerous harmful effects. Simultaneous activation of the coagulation system and the inhibition of fibrinolysis as a result of the pro-inflammatory environment and/or damaged endothelium can result in disseminated intravascular coagulation, which is a major complication of HS, and in the amplification of the inflammatory response. The complement, coagulation and fibrinolysis systems are tightly connected through direct interactions of serine proteases, and imbalances in each cascade are intensified in a positive-feedback loop (Figure 5). Finally, the sustained pro-inflammatory environment affects the functional state of immune effector cells, eventually causing the dysfunction of neutrophils and immunoparalysis. Alterations in leukocyte apoptosis in the later stages of sepsis further account for immunosuppression, which increases the susceptibility to secondary infections. Adapted from Rittirsch, Flierl & Ward, 2008.

Figure 5: **Cross-talk between the complement, coagulation and fibrinolysis systems**

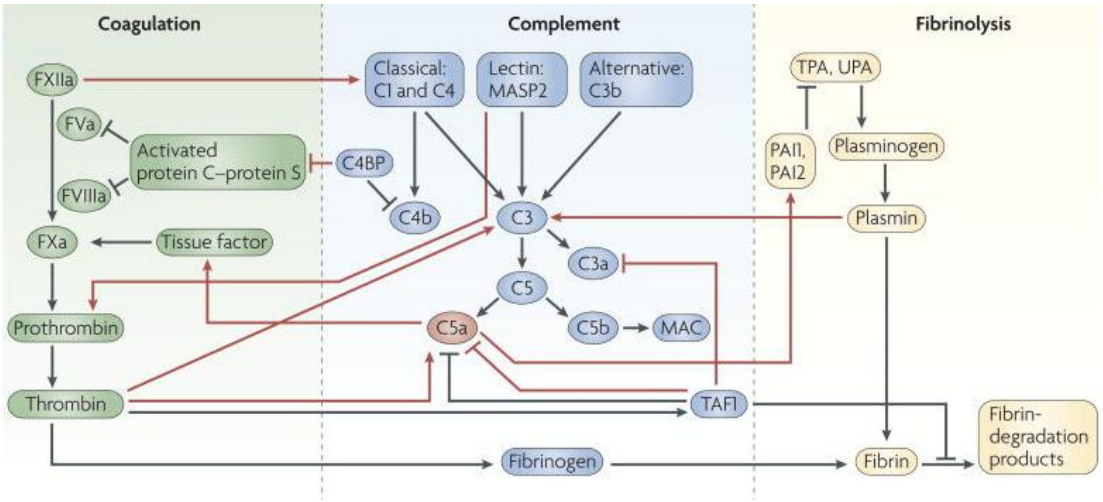

Figure 5: The complement system, the coagulation cascade and the fibrinolysis cascade communicate through many direct and bidirectional interactions (indicated by red arrows). Activated clotting Factor XII can activate the classical complement pathway through cleavage of the complement component C1. Similarly thrombin, kallikrein (not shown) and plasmin directly cleave the complement component C3 and its activation fragments. Moreover, thrombin can cleave C5 into C5a, which occurs independently of C3 and therefore represents a bypass of the three traditional complement-activation pathways (that is, the classical, lectin and alternative pathways). Thrombin-activatable fibrinolysis inhibitor inactivates C3a and C5a in a negative-feedback loop. The complement system also amplifies coagulation through the C5a-mediated induction of expression of tissue factor and plasminogen-activator inhibitor 1 by leukocytes, the latter of which inhibits fibrinolysis. In addition, mannan-binding lectin serine protease 2 of the lectin complement-activation pathway triggers coagulation by converting prothrombin to thrombin. C4b-binding protein of the complement pathway inhibits protein S, which is a co-factor for the activated protein-C pathway of coagulation inhibition, which indicates that the inhibition of anticoagulant mechanisms further augments the pro-coagulant activities of complement. C4BP, C4b-binding protein;FXIIa, Activated clotting Factor XII; MAC, membrane-attack complex (C5b–C9); MASP2, mannan-binding lectin serine protease 2;PAI, plasminogen-activator inhibitor 1; TAFI, Thrombin-activatable fibrinolysis inhibitor TPA, tissue plasminogen activator; UPA, urokinase-like plasminogen activator. Adapted from Rittirsch, Flierl & Ward, 2008.

2.5.1. Cytokines

Cytokines consist in polypeptides or glycoproteins produced by diverse cell types at the site of injury and by systemic immune cells (Lin, Calvano & Lowry, 2000). Contrarily to hormones, cytokines are not stored as preformed molecules and function predominantly by means of paracrine and autocrine mechanisms, although endocrine effects are also possible. Their relatively rapid appearance after injury reflects active gene transcription and translation by the injured or stimulated cell. Cytokines act by binding to specific cellular receptors, leading to the activation of several intracellular signaling pathways that regulate gene transcription.

Through this mechanism, cytokines can influence immune cell activity, differentiation, proliferation, and survival. Cytokines also regulate the production and activity of other cytokines. The capacity of cytokines to activate diverse cell types and to incite equally diverse responses underscores the pleiotropism of these inflammatory mediators. Moreover different cytokines possess similar activity, which means that there is a significant overlap in bioactivity among different cytokines. In addition they can act synergistically or antagonistically in many dimensions. Chemokines are a particular type of cytokines, which are chemotactic meaning that their effects are to attract specific types of immune cells, mainly leukocytes, to the area of injury.

Although a wide variety of cell types are able to produce and secrete cytokines in response to an immune stimulus, the classical view holds that their principal origin is the production and secretion by leukocytes. Cytokines exert powerful effects on many tissues, but cytokines are also major signaling compounds that recruit many cell types in response to injury. Cytokines have effects upon:

- The cells that secrete them- these are autocrine effects;
- Nearby cells- these are paracrine effects;
- Distant cells- these are endocrine effects.

Broadly, cytokines can be classified into four families based on their receptor types:

- Hematopoietins, including IL1 to IL7 and the Granulocyte Macrophage Colony Stimulating Factor group;
- Interferons including interferonα (INFα) and interferonγ (INFγ);
- Tumor Necrosis Factors, including TNFα;
- Chemokines, including IL8.

Soon after formation, Helper T cells differentiate into two types depending from which existing cytokines activate them and then secrete their own cytokines with one of two profiles: Th1, pro-inflammatory; and Th2, anti-inflammatory. Most cytokines classify readily as either Th1 or Th2 according to the influence they exert. For example, IL4 stimulates Th2 activity and suppresses Th1 activity, so it is anti-inflammatory. IL12, on the other hand, promotes pro-inflammatory activity and is therefore Th1. Pro-inflammatory cytokines include IL1β, IL2, IL6, IL8, IL12, IFNγ and TNFα. Anti-inflammatory cytokines include IL4, IL10, insulin-like growth factor 1, and IL13. Some investigators characterize an individual's immune response profile using a Th1/Th2 ratio (Chapman, Tuckett &Song, 2008).

In the following sections three particular cytokines, TNF-α, IL6 and IL10, and its contribution to HS will be discussed.

TNF-α

After acute injury or during infections, TNF-α is one of the earliest and most potent mediators of the host response. HS is no exception and TNF-α has been shown to be the earliest pro-inflammatory mediator to be released in models of trauma/HS (Mi et al., 2011).Depending from the type of experimental model, the increases in TNF-α after HS can be observed in the first 10-45 minutes (Rhee et al., 1993; Ayala et al., 1991; Ayala et al., 1990) and their levels remain elevated in the next several hours (Molina et al., 1997; Ayala et al., 1990; Ayala et al., 1991). However not all studies found systemic elevation of TNF-α levels following HS (Stylianos et al., 1991). In clinical patients this discrepancy has also been found, with some studies demonstrating unequivocally an increase in TNF-α levels following trauma/HS (Namas et al., 2009; Roumen et al., 1993) and others showing the opposite (Foex et al., 1993).

The reason for this discrepancy is incompletely understood. In combined trauma/HS models and clinical studies, the development of HS seems to be pivotal to induce TNF-α release (Schmitz et al., 2010; Roumen et al., 1993; Ayala et al., 1991). However the increase in TNF-α levels seems to be unrelated with degree of tissue injury, the magnitude of the physiological response or the severity of the overall insult (Baker et al., 2012). There is also no correlation between TNF-α concentrations and the degree of haemorrhage (as assessed by the volume of blood transfusion). To further complicate the picture, in experimental studies, it has been demonstrated that even minor interventions such as adjustments in FiO_2 can modulate systemic TNF-α release to the same extent than severe tissue injury or blood loss (Baker et al., 2012). It has been speculated that the differences in systemic levels of TNF-α following HS found in the different studies are related to differences in experimental methodologies. For instances, a recent study demonstrated that volume-controlled and pressure controlled models of HS differ in the amount of cytokine production (Pfeifer et al., 2013). The type of resuscitation fluids is also known to influence the subsequent cytokine response (Watters et al., 2006; Lee et al., 2005). Besides, recent experimental studies demonstrated that HS alone is able to elicit different cytokine responses from traumatic injury and that when trauma and HS are combined, this leads to a blunted cytokine response (Baker et al., 2012; Namas et al., 2009). It has been speculated that the combined insult induces a state of immune paralysis (Hotchkiss et al., 2003). How differences in experimental methodology directly impact different cytokines response is still incompletely understood. Nevertheless it is speculated that different type of injuries impact differently the type and amount of DAMPs release (Baker et al., 2012), which in turn influence the subsequent type of inflammatory response. Finally the differences in cytokine responses in clinical patients

may be due to the huge diversity of individual organ and tissue injuries and the inherent inter-individual variability.

Besides increasing its systemic levels, HS also induces increased tissue TNF-α levels (Jiang et al., 1997; Molina et al., 1997; Tamion et al., 1997). It is known that tissue TNF-α levels do nor correlate with systemic levels (Vallejo et al., 2005; Angele et al., 2008). The tissue increase in TNF-α levels seems to be organ and temporal specific. The main organs which display increased TNF-α levels after HS are the spleen, lung, heart and intestine (Jiang et al., 1997; Molina et al., 1997; Tamion et al., 1997). The first organ where increased TNF-α is observed is the intestine, followed by the liver and the lung (Liu et al., 2007). The increase in tissue levels of TNF-α seems to be regulated by different mechanisms than its increase in systemic levels (Sacoccio, Dornand & Barbanel, 1988). Besides it is believed that it is important for the development of organ dysfunction than the increase in systemic cytokine levels (Vallejo et al., 2005).

Several lines of evidence support the role of TNF- α in HS-associated inflammation. Although its half-life is less than 20 minutes, its brief appearance is sufficient to evoke marked metabolic and hemodynamic changes and activate mediators distally in the cytokine cascade. For instances its diffusion into circulation initiates a state of fatal cardiovascular collapse. Besides, TNF-α triggers the release of other pro-inflammatory cytokines such as IL1 and IL6 (Cai, Deitch & Ulloa, 2010) and may induce MOF through the activation of neutrophils and induction of autolytic inflammation, cell death and apoptosis (Cai, Deitch & Ulloa, 2010; Cairns, Panacek, Harken & Banerjee, 2000; Donnahoo, Shames, Harken & Meldrum, 1999). TNF-α is also a major inducer of muscle catabolism and cachexia during stress by shunting available amino acids to the hepatic circulation as fuel substrates. Other functions of TNF-α include activation of coagulation, promotion of the expression or release of adhesion molecules, prostaglandin E2, platelet-activating factor, glucocorticoids, and eicosanoids.

The intra-signaling pathways that are involved in TNF-α secretion and production are becoming to be fairly elucidated. Oxidants, DAMPs and other stimulus released following ischemia and/or reperfusion, activate p38 MAPK and the TNF transcription factor, NF-κB, leading to subsequent TNF synthesis (Donnahoo et al., 1999). The TNF-α which is released binds to specific TNF membrane receptors which will further reactivate the NF-κB pathways (Cairns et al., 2000). With this mechanism TNF can upregulate its own expression as well as facilitate the expression of other genes involved the inflammatory response. The pivotal role for NF-κB associated increase in tissue TNF-α mRNA and serum TNF- α levels has been demonstrated in an experimental model of HS demonstrated (Altavilla et al., 2001).

The actions of systemic TNF-α are regulated by circulating soluble TNF receptors (sTNFRs). sTNFRs are proteolytically cleaved extracellular domains of membrane-associated TNFRs

that are elevated and readily detectable in acute inflammation. sTNFRs retain their affinity for the binding of TNF-α and therefore compete with the cellular receptors for the binding of free TNF-α. sTNFRs thus represent an endogenous counter-regulatory response to excessive systemic TNF-α activity. They also serve as a carrier (eg, transporter) or as a storage pool of bioactive TNF- α in the circulation.

The major stimulus that triggers increased TNF-α production and secretion in HS has been the subject of intense investigation. Based in what has been described for septic shock, for some time, endotoxin was initially proposed to be the major stimulus for inducing TNF-α ´s production, release and subsequent development of MOF (Deitch, 1992). In this theory the source of endotoxin was its translocation from the intestine, after HS-induced increased intestinal permeability (Wang, Ba, Cioffi, Bland & Chaudry, 1998; Roumen et al., 1993). Subsequently, several studies evaluated if increased TNF-α levels were correlated with the levels of tissue endotoxin. This was found in some studies (Jiang et al., 1997; Jiang et al., 1995), but not in others (Kelly et al., 1997; Endo et al., 1994; Ayala et al., 1990). In addition it was found that HS is associated to lower systemic levels of TNF-α than septic shock (Foëx et al., 1997; Endo et al., 1994). Furthermore, experimental studies demonstrated that HS and LPS were associated to different TNF-α response (Molina &Abumrad, 2000).Currently it is believed that although endotoxin may play a role in increasing TNF-α following HS, there are other stimuli which are also involved. These include tissue ischemia/hypoxia (Ertel et al., 1995; Ghezzi et al., 1991), oxidant and I/R injury (Tamion et al., 2000; Tamion et al., 1997) and the release of DAMPs from dying cells. Important DAMPs which were particularly associated to increased TNF-α levels after HS are mitochondrial proteins (Chaung et al., 2012; Bianchi & Manfredi, 2007) and HIF-1 (Feinman et al.¸ 2010). Blood pH was also found to be negatively correlated with TNF-α (Feinman et al.¸ 2010). The latter most probably reflects the association between the degree of metabolic acidosis caused by tissue ischemia and injury and the increase in TNF-α.

The primary sources of TNF-α synthesis include monocytes/macrophages and T cells, which are abundant in the peritoneum and splanchnic tissues. In HS, it is believed that TNF-α is mainly produced by innate immune cells, especially macrophages present in the GI tract, (Tamion et al., 2000; 1997; Deitch et al., 1994), spleen (Molina, 2001) and peritoneum (Zhu et al.¸1994). Other cellular sources include endothelial cells (Tamion et al., 2000; Deitch et al., 1994), hepatic Kupffer cells (Ayala et al., 1992), and blood monocytes (Rhee et al., 1993). Gastrointestinal ischemia may be particularly relevant in this context. During HS the GI tract is particularly susceptible to ischemia and/or I/R injury (Davidson et al., 2004). This leads to increased local TNF-α production which subsequently gains access to the systemic circulation through the mesenteric lymph and the portal vein. Through this way TNF-α and

other inflammatory mediators reach the lung, leading to tissue injury (Cai, Deitch & Ulloa, 2010).

If TNF- α has a beneficial or detrimental role in HS is still being debated. Early studies have implicated a pivotal role of the cytokine for the development of MOF (Bahrami et al., 1997; Rhee et al., 1993). However, attempts to modulate the effects of TNF-α in the setting of Trauma/HS have had mixed results. Bemelmans et al*.,* (1993) found that administering anti-TNF-α antibodies to jaundiced mice subjected to surgical trauma was not associated with improvement in survival (Bemelmans et al., 1993). In contrast mortality of wild-type mice subjected to HS was decreased by pre-treatment with anti-TNF-α antibodies (Demaria, Pellicane & Lee, 1993). Zingarelli et al*.*, (1994) found that anti-TNF-α antibodies improved survival in an extremely severe model of HS in rats (death by 30 minutes post-hemorrhage). Furthermore, various studies also suggested improvements in histological parameters following treatment with anti-TNF-α in the settings of T/HS, but did not document effects on survival. For example, Marzi et al*.*, (1995) found that anti-TNF-α antibodies attenuated leukocyte adhesion in the livers of rats subjected to HS, and Abraham et al., (1995) found evidence of reduced lung inflammation (Abraham et al., 1995; Marzi et al., 1995). However more recently in a pig model of HS, an early and robust TNF-α response was found to be essential to survival (Namas et al., 2009). In fact, the authors found an inverse correlation between TNF-α production and organ damage/dysfunction, suggesting that early TNF-α serve either to limit organ damage or to induce reparative processes. It might be possible that early in HS, TNF-α functions as an alarm-phase cytokine, indicating a self-limiting form of inflammation that signals for healing of injury and that only when its levels are pathological increased or extended, its pathological role becomes apparent.

IL6

Several studies, both clinical and experimental, have also demonstrated that HS is associated to increased systemic levels of IL6 (Batistaki et al., 2008; Liu et al. 2007; Roumen et al. 1993; Bitterman et al., 1991; Ayala et al., 1991; Ayala et al., 1990). HS also increases local tissue levels of IL6 (Liu et al., 2007; Jiang et al., 1997; Molina et al., 1997; Tamion et al., 1997).

Contrary to TNF-α, the systemic levels of IL6 were found to be of diagnostic and prognostic value. An increase in IL6 levels is diagnostic for the presence of HS and predicts the need for massive blood product substitution in this setting (Sapan et al., 2016; Bogner et al., 2009). IL6 is actually considered the best biomarker of outcome of trauma patients with Systemic Inflammatory Response Syndrome (SIRS), sepsis, and Multiple Organ Failure (MOF) (Pape et al., 2007; Remick et al., 2002; Peitzman et al., 1995; Roumen et al., 1993).

The role of IL6 in HS is complex. Insights from several computational studies suggested that the cytokine may be involved in a positive feedback loop of inflammation, which induces tissue dysfunction/damage which subsequently set the stage for more inflammation (Chow et al., 2005). Nevertheless and in contrast to sepsis (Remick et al., 2002), early elevations of IL-6 may play a prominent role in the response to T/HS, especially in the inflammatory process following resuscitation in HS (Meng et al., 2001). Interestingly, later elevations are associated with increased morbidity and persistent elevations in IL-6 may be indicative of self-sustaining, tissue-damaging inflammation (Sperry et al., 2008; Maier et al., 2007).

In the initial stages of injury, IL6 is an important mediator of the hepatic acute-phase response, inducing hepatic acute phase response protein synthesis with release of C-reactive protein and procalcitonin (Brøchner& Toft, 2009). It also activates neutrophils and NK-cells. However it delays the phagocytic disposal of senescent or dysfunctional neutrophils, thereby prolonging the injurious effects mediated by these cells (Brøchner& Toft, 2009). In several hours post-injury, IL6 changes its function and begins to display an anti-inflammatory phenotype. IL6 then stimulates the release if IL1 receptor antagonists and of sTNFRs, prostaglandin E2 and IL10 (Brøchner & Toft, 2009; Meng et al., 2001). Contributing to its anti-inflammatory effects at this stage, IL6 is a potent stimulus of the HPA axis, leading to increased CS levels (Bethin, Vogt & Muglia, 2000). Although the mechanisms behind the change in IL6´s phenotype are still incompletely understood, it is known that at least partially they derive from changes at a transcriptome level (Moran et al., 2011). Interestingly it seems that at a local level, IL6 maintain its pro-inflammatory role.

There are significant differences between the release of IL6 and TNF-α in HS. The first is that, contrarily to TNF-α, soft tissue trauma seems to be a potent stimulus of IL6 secretion (Foex et al., 1993; Roumen et al., 1992; Ayala et al., 1991). For instances Ayala et al., (1991) found that despite IL-6 increased continuously post-hemorrhage, its levels were already increased after midline laparotomy and before initiation of hemorrhage compared with non-manipulated animals. By contrary, in the same study, TNF-α was only detected after hemorrhage was initiated (Ayala et al., 1991). In addition IL6 levels seem to be proportional to the severity of tissue injury (Roumen et al., 1993). In contrast TNF-α is mainly induced by HS, and there is a threshold for overall injury that must be exceeded before TNF-α elevations are observed after tissue injury (Baker et al., 2012; Namas et al., 2009). Second, experimental studies have shown that IL6 secretion lags behind and tends to last longer than TNF-α secretion (Liu et al., 2007; Ayala et al., 1991).In fact, IL6 levels begin to be detectable in the circulation at 60 minutes after injury, peak between 4 and 6 hours, and can persist for days (Sapan et al., 2016; Liu et al., 2007; Roumen et al., 1993).Because the half-life of IL6 is just about 60 minutes, this indicates that the cytokine must be continuously produced for its levels to remain elevated (Biffl et al., 1996).

The stimuli that induce IL6 secretion after HS are still incompletely understood. Some authors consider soft tissue injury as the predominant stimulus for IL6 (Ayala et al., 1991). Other known stimulus include hypoxia (Ertel et al., 1995; Yamauchi-Takihara et al., 1995), ischemia (Hierholzer et al., 1998); DAMPs (Bianchi, 2007; Bianchi & Manfredi, 2007), and I/R injury (Meng et al., 2001; Hierholzer et al., 1999; Hierholzer et al., 1998; Yamauchi-Takihara et al., 1995; Deitch et al., 1994). Interestingly, although TNF-α and IL1 are potent inducers of IL6 production from virtually all cells and tissues (Cai, Deitch & Ulloa, 2010), trauma and HS, the IL6 response to injury appears to be independent of these cytokines (Biffl et al., 1996).

IL10

IL10 has also been shown to be increased following trauma and HS (Brøchner & Toft, 2009; Namas et al., 2009; Schneider, Schwacha & Chaudry, 2004; Sherry et al., 1996). IL10 is a pleiotropic cytokine produced by both T/B cells and macrophages which possesses both anti-inflammatory and immunosuppressive properties (Moore et al., 1990). Extensive research have shown that IL10 is an inhibitor of a broad spectrum of monocyte/macrophage functions, including cytokine synthesis, NO production, and expression of MHC class II and co-stimulatory molecules such as CD80/CD86 (Moore et al., 2001).

Investigations in numerous inflammatory disease models including chronic enterocholitis, cutaneous inflammatory conditions, endotoxic shock, Shwartzman reaction, and autoimmune encephalomyelitis in $IL10^{-/-}$ mice have yielded strong evidence that IL10 plays a central role in restricting inflammatory responses *in vivo* (Fuss et al., 2002). In addition, IL10 also processes immunostimulatory effects. It is a potent growth factor for B lymphocytes, promotes B-cell proliferation, antibody production, and class II expression (Schall et al., 1990). IL10 also enhances the development of cytotoxic T lymphocytes (Macneil et al., 1990), induces NK cytotoxicity against NK-resistant tumor cells *in vitro* and increases IL2-induced NK cell proliferation (Carson et al., 1995). In addition it acts as a cofactor for colony formation by mast cell progenitors (Robinson et al., 2003) and thymocytes (Macneil et al., 1990).

There is evidence that the early secretion of IL10 after HS modulates the magnitude of the pro-inflammatory response through a paracrine way in order to prevent that this becomes excessive (Schneider, Schwacha & Chaudry, 2004; Yokohama et al., 2004). This beneficial role has been demonstrated in experimental models of endotoxemia, where IL10 was mainly associated to the modulation of TNF-α levels (Marchant et al., 1994; Smith et al., 1994; Howard et al., 1993). Furthermore Cui et al., (2005) reported that part of the beneficial effects of adrenomedullin in rats with HS were due to its ability to increase IL10 levels (Cui et al., 2005)

However, excessive increases in endogenous IL10 production can also exacerbate macrophage and T-cell dysfunction, decrease T-cell apoptosis, blunt antimicrobial activity, and increase mortality. This has been demonstrated in acute bacterial models of sepsis and in thermal injury (Oberholzer, Oberholzer & Moldawer, 2002). Moreover, it has been demonstrated that IL10 is immunosuppressant in animal models of trauma and HS (Ayala et al., 1994) and that the increase in IL10 and other T_H2 cytokines is currently though to play a major role in development of immunosuppression and sepsis after trauma (Delong & Born, 2004).

Experimental studies have shown that IL10 increases begins in the first 15 minutes post-hemorrhage, and peaks between 60 to 120 minutes thereafter (Baker et al., 2012; Schneider, Schwacha & Chaudry, 2004). The main cellular origin of IL10 in HS is believed to be Kupffer cells in the liver (Schneider, Schwacha & Chaudry, 2004). IL10 release seems to be induced by TNF-α, IL1 and IL6 (Brøchner & Toft, 2009), glucocorticosteroids (Elenkov, 2004) and directly by endogenous DAMPs (Maslanik et al., 2013, Levy et al., 2007). In addition there is evidence that the increased production of catecholamines which accompanies HS can be responsible for the production of IL10 at the late stages of HS (Namas et al., 2009; Batistaki et al., 2008; Platzer et al., 2000; Woiciechowsky et al., 1998).

2.5.2. The complement system

The importance of the complement system in the pathophysiology of several critical illnesses has been increasingly recognized in recent years. The complement system is an ancient defense system, composed by more than 35 effectors and regulators which play a central role in innate immunity. In Humans, the complement system is immediately activated after trauma and the plasma levels of complement components are directly correlated with the severity of injury and inversely with the outcome (Burk et al., 2012).

The complement system can be activated by three pathways: the classical, alternative and lectine pathways and its major functions are pathogen elimination and immediate response to danger signals (Surbatovic et al., 2013). However the complement cascade may also lead to deleterious effects by mounting an attack on host tissue as it has been reported in I/R injury (Surbatovic et al., 2013). Complement also exacerbates local and systemic inflammation and contributes to the release of toxic mediators, thus increasing the “antigenic load.” It is also known that one of its products, the anaphylatoxin C5a leads to detrimental effects on the host’s immune system (e.g. neutrophil dysfunction and immunoparalysis, thymocytes apoptosis and coagulopathy). All these effects may justify why complement activation has been associated to development of MOF (Rittirsch, Redl & Huber-Lang, 2012)

and why deficiency in complement products, and in particular of C3 has been shown to improve outcome in HS (Cai et al., 2010).

2.5.3. The coagulation system

The coagulation system is also activated as a consequence of tissue injury, immune cell activation and pro-inflammatory cytokine release and coagulation derangements are frequently found in cases of hemorrhage and inflammation (Hook & Abrams, 2012; Levi, Van der Poll & Schultz, 2012). In fact the relationship between any type of insult to the body, external or internal, and activation of the coagulation system is an ancient phylogenetical one. It may be said that the localized activation of the coagulation system associated to the innate inflammatory/immune response and local and systemic hemodynamic reflexes has a protective role against any insult and aims to prevent blood loss and damage from alien substances.

Several examples exist that demonstrate the existence of an extensive cross-talk between the coagulation and immune systems (Rittirsch, Flierl & Ward, 2008). For example activated coagulation proteins such as thrombin, factor Xa and tissue factor VIIa can stimulate cytokine production, which in turn stimulate the coagulation cascade. For example the cytokines TNF-α and IL6 stimulate the production and release of tissue factor by endothelial cells and monocytes and tissue factor is a known activator of the coagulation cascade (Hook & Abrams, 2012). Coagulation factors also increase vascular permeability and produce vasodilatation; are chemotactic and enhance leucocytes adhesion by increasing the expression of adhesion molecules and platelet activating factor on intravascular cells; activate the intravascular tissue factor expression and downregulate the fibrinolytic and protein C anticoagulant pathways (Bonanno, 2011). Activated platelets can secrete chemokines, express adhesion molecules on the endothelium and promote neutrophil adherence. Complement activation products amplify coagulation and inhibit fibrinolysis mainly through C5a, which induces the expression of tissue factor and plasminogen activator inhibitor 1. In addition, mannose-binding protein associated serine protease 2 activates both the complement and coagulation cascades, generating thrombin. Vice versa, activated clotting factor XII triggers the classical complement pathway via C1 cleavage, whereas thrombin directly cleaves C5, generating biologically active anaphylatoxin C5a.

The body devised several mechanisms to control excessive coagulation system activation. The three major natural anticoagulant mechanisms are the production of antithrombin, activated protein C and tissue factor pathway inhibitor (Hook & Abrams, 2012). Normal endothelium cells also release other compounds which have anticoagulant activity. These include NO and prostacyclin which not only control vascular tone but also have antiadhesive

and profibrinolytic properties by promoting tissue plasminogen activator release. In addition there are several membrane-associated components which have anticoagulant properties expressed in the outer membrane of endothelial cells including protein C, protein S, thrombomodulin and heparan sulfate. Another protective mechanism is that when some components of the coagulation cascade are activated, they simultaneously initiate the fibrinolytic pathway. For example, factor XII formation triggers the intrinsic coagulation cascade which leads to the formation of thrombin, fibrinogen, fibrinopeptides and fibrin and simultaneously fibrinolytic system activation with end formation of plasminogen, the fibrinolytic plasmin and fibrin degradation products.

In severe critical illnesses such as HS and sepsis, a shift for the procoagulant state occurs, due to several mechanisms including increased procoagulant activity, decrease in fibrinolytic activity, and decreased concentration of natural anticoagulants. Increased procoagulant activity is in part related with the increased pro-inflammatory cytokine typical of these conditions, which leads to increased expression of tissue factor by endothelial cells and monocytes and increased platelet activation (Hooks & Abrams, 2012; Esmon, 2004). In addition, in HS the presence of vascular injury exposes tissue factor presents in tissues to blood, which also initiates the coagulation cascade. The procoagulant state also occurs due to depletion in anti-coagulant systems, due to rapid consumption by accelerated coagulation, decreased (e.g: antithrombin is an negative acute phase protein) or impaired hepatic synthesis, removal via the reticuloendothelial system, degradation by neutrophil elastase and leakage from capillaries (Levi, Van der Poll & Schultz, 2012). Pro-inflammatory cytokines also decrease the synthesis of thrombomodulin and of glycosaminoglicans in the endothelial cell´s surface. The lack of glycosaminoglicans contribute to decreased antithrombin function as they act as heparin-like factors for antithrombin activity (Levi, Van der Poll & Schultz, 2012). Finally a decreased fibrinolytic activity occurs due to increase production of substances such as plasminogen activator inhibitor type 1 and α1-plasminogen inhibitor, which may be caused by the action of pro-inflammatory cytokines and also bacterial endotoxin. The end result of impaired fibrinolysis is inadequate fibrin removal, contributing to persistence of the microthrombi (Hooks & Abrams, 2012).The shift to the procoagulant state is associated to widespread platelet aggregation and fibrin deposition throughout the microcirculation, which lead to the formation of microthrombi (Levi, Van der Poll & Schultz, 2012). These may eventually lead to ischemia and contribute to MOF (Gando, 2010). In addition due to consumption of platelets and coagulation factors, a state of disseminated intravascular coagulation (DIC) may occur (Hooks & Abrams, 2012). DIC with diffuse paradoxical oozing due to consumption coagulopathy by the disseminated microthrombi is a prognostic marker of sepsis indicating a late-stage critical illness, and becomes a sign of irreversibility if persists despite treatment.

In resume the first phase of the reaction to HS-induced damaged is characterized by leukocyte activation, ROS production due to I/R injury, inflammatory mediator´s release (cytokines, NO), activation of the complement, coagulation and kallikrein/kinin cascades and endothelial cell damage.

2.6. The response to HS spreads-the initial localized inflammatory response (LIR)

The initial molecular events and subsequent cellular injury which follow HS´s first hit correspond to the most early and fundamental body´s reaction and constitute the foundation for the next stage: the development of a LIR. The LIR is only present when hemorrhage induces the first hit because its effectors reach out the systemic circulation through the solution of continuity which is produced in the vessels walls. However it rapidly follows and it is characterized by the development of microvascular and cellular changes which aim to bring neutrophils (the main effector cells at this stage) and other cellular and humoral factors into the damaged or injured area. Interestingly these cellular and humoral factors (inflammatory mediators) not only initiate the LIR but are also able to amplify *in situ* the inflammatory phenomenon and reproduce it distally in systemic fashion.

The vascular changes induced by the LIR are mainly characterized by arteriole vasodilatation, increased hydrostatic pressure, flow stasis, increased microvascular permeability and hemorrhage. An ultrafiltrate of plasma constituted by water and few small diameter proteins passes into the injured area, creating a local transudate. Three types of microvascular responses have been identified: an immediate transient response involving only venulae resulting from endothelial cell's contraction; an immediate persistent response involving all the microcirculation components (arterioles, capillaries and venulae) caused by direct endothelial damage; a delayed persistent response involving capillaries and venulae due to slow onset endothelial cells damage (Bonnano, 2011). Initially the vascular changes may result from direct damage to endothelial cell´s by hypoxia, I/R injury, loss of endothelia's glycocalix, damage to the basement membrane and the production of inflammatory mediators. Key mediators include the cytokines TNF-α and IL1, IL6, IL8 and IL12. Release of TNF-α and IL1 are early events, whereas later production of IL6 and IL8 prolongs the inflammatory response. Many of the classical features of inflammation can be attributed to TNF-α through induction of iNOS and COX-2, leading to vasodilation, increased capillary permeability, and local slowing of blood flow. In addition a direct disruption of endothelial barrier function is a direct consequence of cytokine production. Cytokines such as TNF-α and bradikins and prostaglandins are known to increase vascular permeability. Although the changes in microvascular tone following HS are still incompletely understood, mediators

such as NO, endothelin, catecholamines and arginine vasopressine (AVP) seem to be key actors in that response (see later–microvascular response to HS).

A critical development in LIR occurs when leukocytes, especially neutrophils, adhere to the endothelium. This process begins when leukocytes marginate and roll over the endothelium, where they initially establish a loose connection with endothelial cells. Inflammatory substances such as leukotrienes and prostaglandins which result from the catalytic activity of phospholipase A_2, complement components and cytokines induce the recruitment of leukocytes to injured tissues. Early inflammatory cytokines such as TNF-α and IL1 also induce the expression of adhesion molecules in the endothelial surface and also activate neutrophils. Once activated, neutrophils have an upregulated expression of adhesion molecules in their surface. These recognize the correspondent molecules in the endothelial cell´s surface permitting adhesion of neutrophils to the endothelial cells. Neutrophils release ROS, proteolytic enzymes and other lipid mediators which further increase tissue injury (Rose & Marzi, 1998). In the endothelium, these substances promote further microvascular injury, by affecting cell-to-cell junction and destroying the endothelial glycocalyx, leading to tissue edema and further oxygen extraction deficit (Surbatovic et al., 2013). Locally, they promote cell death by necrosis, release of cytosolic and nuclear components, and degradation of proteoglycans in the extracellular matrix. These actions unmask multiple new DAMPs, accelerating innate immune system activation and resulting in the production of yet more proinflammatory cytokines/chemokines in a vicious cycle of tissue injury and inflammation. It is now recognized that the inappropriate activation and positioning of neutrophils within the microvasculature is pivotal for the pathological manifestations of MOF (Brown et al., 2006). It is also known that the interaction of endothelial cells or leukocytes with platelets, after these have become activated and aggregated also increases the complex network of cellular interactions and further mediator production (Rose & Marzi, 1998).

Interestingly it is known that the chain of events that characterize the LIR and which was described above, changes slightly accordingly to the anatomical place of injury. This concept is known as compartmentalization of the inflammatory response (Cavaillon & Annane, 2006). Accordingly to this, the nature of the insult (e.g. burn, hemorrhage, trauma, peritonitis), the type of cells present in each compartment (e.g. type and number of phagocytes, characteristics of endothelial cells), and its micro-environment (e.g. local presence of granulocyte-macrophage colony stimulating factor in the lungs, low levels of arginine in the liver, release of endotoxin from the gut), and leukocyte recruitment, have a great influence in the magnitude and characteristics of the LIR and subsequent tissue injury. The effects of extracellular mediators and the responsiveness of inflammatory cells are also dependent from injury location (circulating vs fixed tissues) (Cobb & O´Keefe, 2004). A particular

example of the concept of compartmentalization is that in the lung and liver leukocyte adhesion to endothelial cells occurs independently from adhesion molecules. This may explain why lesions in these organs are more frequently found in critically ill patients than myocarditis, for example (Bonnano, 2012).

Ultimately, the LIR aims to induce the healing of injured tissues and to limit/control the invasion of foreign organisms. In an effort to contain the inflammatory reaction to the site of injury, the body initiates concomitantly a systemic anti-inflammatory response. However if the local pro-inflammatory response becomes excessive in magnitude and extent and/or the anti-inflammatory containment fails, then a systemic generalization of the inflammatory response occurs. As we will see, this systemic generalization leads to dysfunction of vital tissues distant from the original place of injury (Surbatovic et al., 2013; Brøchner & Toft, 2009; Hierholzer & Billiar, 2001).

2.7. The global reaction to HS

Besides the local intracellular, cellular and tecidual response, HS also initiates a systemic adaptive response which involves a synchronized interaction between the neuronal, immune and endocrine systems with the aim of restoring homeostasis (Molina et al., 2006) (Figure 6). In fact, HS triggers what it is generally known as the defense response. The term defense response refers to the complex response that involves the sensorial detection and multi-subsystem, self-organizing arousal to tissue injury or threat of tissue injury (Chapman, Tuckett & Song, 2008). Generally speaking, the defense response induces physiological changes to facilitate three possible solutions to the threat symbolized by the stressor: to fight, to flight or to freeze.

The defense response involves adaptive actions at multiple levels including neurologic (threat detection in the external environment, cognition with anticipation and appraisal, signaling of the incurred injury, and motor responses geared to escape or fighting), endocrine (the stress response), cardiovascular/respiratory and immune (microbial invasion and toxin production detection and activation of inflammation to protect against microbial threat and to promote wound healing). Thus, the defense response involves the purposeful and coordinated activity of the endocrine, immune and nervous subsystems. Some authors even consider that the three interdependent subsystems actually function as single overarching system or supersystem, which acts in a coordinated manner to obtain allostasis (Chapman, Tuckett & Song, 2008). In this case allostasis is referred to the protective, coordinated, adaptive reaction to maintain homeostasis (Chapman, Tuckett & Song, 2008). In the following sections the global response to HS will be discussed in its subcomponents. The immune response will be firstly described, followed by the cardiovascular response (which

due to the nature of the stressor that is HS it is particularly relevant) and finally the endocrine response, with emphasis in the HPA and thyroid axis.

Figure 6: the global reaction to HS

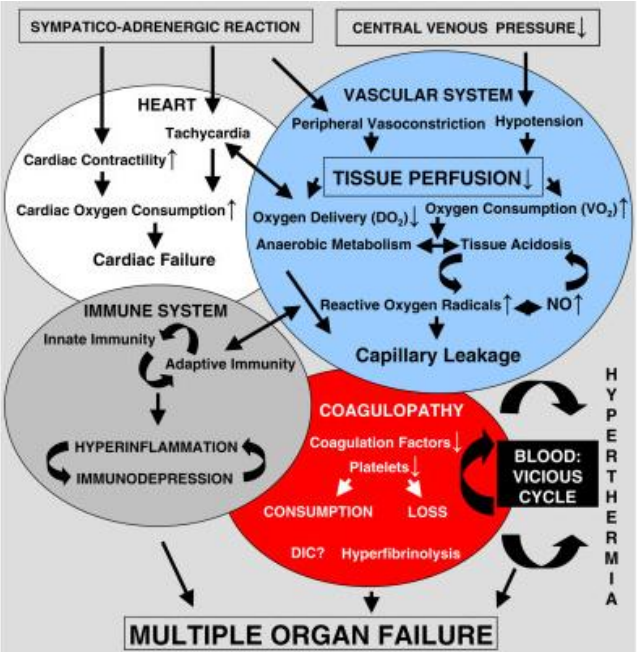

Figure 6: HS induces a response which coordinates the endocrine, cardiovascular and immune systems. If these systems fail to restore hemostasis the result is MOF. Adapted from Angele et al., 2008

2.7.1. The global immune response to HS

It was already mentioned that HS induces the activation of the innate immune response at tissue level. It is now understood that this is accompanied by a simultaneous downregulation of the adaptive immune response. This occurs very early at a genomic level, although it only becomes manifest few days later (Xiao et al., 2011). This down-regulation of the adaptive immune response is believed to be one of the underlying causes for the increased risk of infection that develops in hospitalized trauma and septic patients (see below).

When the LIR to HS becomes exaggerated and/ or uncontrolled, it spreads systemically, in a process called SIRS (Angele et al., 2008). SIRS is than defined as the body´s systemic inflammatory response to a variety of severe clinical insults and it manifested by at least two of the following features: hyperthermia or hypothermia; tachycardia; tachypnea or hyperventilation and leukocytosis or leukopenia (Robertson & Coopersmith, 2006). The development of SIRS can lead to tissue destruction in organs not originally affected by the initial injury leading to subsequent development of MOF (Surbatovic et al., 2013; Brøchner & Toft, 2009; Hierholzer & Billiar, 2001).

The currently accepted dogma states that in conditions such as sepsis and HS, following the pro-inflammatory state of SIRS´s, a state characterized by immunosuppression and

susceptibility to infection called compensatory anti-inflammatory response (CARS) than occurs. Accordingly to this concept, CARS develops as an attempt of the body to refrain/control the hyperinflammatory SIRS. The biological effectors of SIRS and CARS are than pro and anti-inflammatory mediators, respectively andmost of them are cytokines. There are also specific immune cells involved in each stage. In patients which survive, following a moderate SIRS state a limited CARS develop and eventually, as disease resolves, the immune system reaches the original equilibrium. In this view, the worse phenotypes occur when SIRS is severely exaggerated, leading to excessive tissue destruction and MOF or when CARS is also pathologically amplified, leading to development of severe secondary and fatal infections.

2.7.2. SIRS and CARS

SIRS is characterized by is the excessive production and release of pro-inflammatory cytokines such as TNF-α, IL6 and IL1. It is also accompanied by polymorphonuclear neutrophil activation and microvascular adherence, and uncontrolled neutrophil and macrophage oxidative burst. The massive release of pro-inflammatory cytokines such as TNF-α and IL6 after HS has been demonstrated both in rats (Ayala et al., 1991; Bitterman et al., 1991) and Humans (Roumen et al., 1993).The massive and continuous IL6 release induces the acute phase response. However, more importantly, also accounts for the up-regulation of major anti-inflammatory mediators, such as prostaglandin E2, and the anti-inflammatory cytokines that characterize CARS, namely IL10 (Yokoyama et al., 2004) and transforming growth factor β (TGF- β) (Cai, Deitch & Ulloa, 2010).

CARS is characterized by immunosuppression, which is indicated by immune cell hypoactivity and a shift of the lymphocyte Th1/Th2 ratio towards a Th2-dominated cytokine pattern. Examples of immune cell hypoactivity which are characteristic of CARS are monocyte deactivation (reflected by the lack of monocytic TNF-α production upon LPS stimulation), impairment of phagocytic capacity and generation of ROS by neutrophils (regardless the fact that they present surface markers of activation such as increased levels of CD11b and CD64), and macrophage dysfunction. There is also decreased bacterial clearance, decreased capacity to present antigens and decreased ability to release proinflammatory cytokines. In sepsis, the presence of defective T cells, with apoptotic depletion and decreased lymphocyte proliferation have also been identified.

Much of the view of the immune response to HS as a state which alternates between SIRS and CARS was based in experimental models of HS and in data obtained from other critical illnesses, including trauma, burns, and sepsis. Recent genomic and clinical studies have now shown that the immune response to critical illness is far more complicated than the simple

mechanistic view where SIRS is followed by CARS. In fact we now know thatboth the pro-inflammatory and anti-inflammatory responses are activated very earlier in the disease course. This activation occurs simultaneously and begins at the genetic level (Xiao et al., 2011). In evolutionary terms, the activation of both pathways makes sense. In the currently accepted view, pro-inflammatory pathways are activated to fight/control offending pathogens and to initiate healing of the injured tissues. Simultaneously, the anti-inflammatory pathways are activated to contain inflammation to the site of injury, where it is needed, thus preventing its spread to distant tissues.

It is now recognized that isolated hyperinflammation or immunosuppression are not the only pathological phenotypes which can occur following HS and that this equilibrium between pro-inflammation and ant-inflammation can be disturbed in several ways. For example many critically ill patients show signs of persisting inflammation and immune-mediated organ damage while simultaneously remaining highly susceptible to secondary infections, suggesting that bot pro and anti-inflammatory tendencies are overactivated. Patients with this phenotype have been said to be under a state called complex immune dysfunction syndrome (CIDS) (Surbatovic et al., 2013). In other words, after their initial injury, these patients also presented signs of immune mediated damage at organs distal from the initial site and simultaneously a global immunosuppressive state which increases the risk of developing secondary infections, especially in the urinary and respiratory tracts. Others authors came with the suggestion of designating the complex immune changes that affect critically ill patients with late MOF as persistent inflammation and immuno suppression catabolism syndrome (Vanzant et al., 2014).

In resume, recent findings suggest that the immune response to HS seems to be initially characterized by a pro-inflammation phenotype at the place of injury, and simultaneously by a systemic anti-inflammatory state which aims to contain inflammation to where it is needed to prevent additional injury. However several factors can change this equilibrium. The inflammatory process may become systemic, leading to organ damage distant to the initial injury site or the immunosuppression may become severe enough that allows the pro-inflammatory phenotype unrestrained or increase the risk for development of secondary infections. The factors which underlie this disequilibrium are not yet completely understood, although they may include the location, type and extent of injury, the presence of comorbidities and of specific host factors such as age and genetic predisposition. Regarding the latter, it is interesting to note that a correlation between clinical and immune phenotype and sepsis was confirmed by genetic mapping of single nucleotide polymorphisms present in the genes of IL6, IL18, TNF-alpha, IFNγ, and TLRs (Giannoudis, van Griensven, Tsiridis & Pape, 2007; Reid, Perrey, Pravica, Hutchinson & Campbell, 2002). The presence of a secondary injury, the so-called “second-hit” (e.g. surgery and hemorrhage) can also

contribute for its development, as they represent additional sources of DAMP´s and PAMP´s. Simultaneously, the development of infection following the immunosuppressive state represents an additional factor that initiates further cycles of inflammation/anti-inflammation.
This being said, it maybe be considered that our tendency to explain the immune response to HS as a continuous process, from LIR to SIRS and than from this to CARS is probably incomplete and far more complex. It is possible that the immune response to HS can evolve into different scenarios which demand different clinical approaches . Some authors begin to apply this line of reasoning for sepsis. For example Hotchkiss and coworkers proposed that the immune response to sepsis follows three possible scenarios. The first scenario occurs characteristically in previously healthy patients with severe sepsis. In this patients, the initial phase is characterized by an excess hyperinflammatory-proinflammatory response with fever, hyperdynamic circulation, and shock, although both proinflammatory and anti-inflammatory responses begin rapidly and concomitantly after sepsis onset. In this scenario, patients mostly die from cardiovascular collapse, metabolic derangements, and MOF. For these patients, the best therapeutic approach would consist in the administration of a short acting anti-inflammatory or anticytokine therapy. The second scenario is more frequent in older patients which present several comorbidities affecting their immune response. These patients are characterized by a blunted or absent hyperinflammatory response, but maintain and even present an augmented anti-inflammatory phase. Consequently, their major cause of death is the development of secondary infection. In these cases the best therapeutic approach would to boost immunity with immunoadjuvant therapy. Finally, in the third scenario, patients have an immune response which cycle between hyperinflammatory and hypoinflammatory states. Initially they experience a hyperinflammatory response, followed by hypoinflammatory state. With the development of a new secondary infection, patients experience a new onset of hyperinflammatory reaction and may either recover or reenter in the hypoinflammatory phase. Death may occur in either state. The longer the sepsis continues, the bigger the chances that the patient has to develop profound immunosuppression.

2.8. The cardiovascular response to HS

The cardiovascular response to hemorrhage follows three distinct phases: an initial phase of tachycardia and increased vascular resistance which aims to maintain arterial pressure; a second phase characterized by hypotension and bradycardia; a third phase, which occurs when blood loss exceeds 44% of the total blood volume, and which is characterized by a massive increase in heart rate and a further reduction in blood pressure (Foëx, 1999). These phases result from the activation of several reflexes which in turn are activated by changes

induced by HS, in particular the decrease of blood pressure and oxygen content. In this regard the three most important cardiovascular reflexes are the arterial baroreceptor reflex, the “depressor reflex” and the chemoreflex. These and others will be discussed briefly in the following sections.

Baroreflex in HS

The afferents of the baroreflex are arterial baroreceptors located in the aortic arch, lung and carotid sinus (Ganong, 2005). At blood pressures comprehended between 50-60 and 160-180 mmHg, baroreceptor receptors discharge basally and continuously via vagal and glossopharyngeal nerves afferent sensory pathways, to the cardiac and vasomotor centers in the medulla oblongata. The cardiac and vasomotor centers are the portions of the Central Nervous System (CNS) that control the basal tone of the entire circulation (heart, arterioles and venules). This control is exerted through the parasympathetic vagal nerves which modulate heart rate and through the sympathetic spinal cord-peripheral nerves which control the basal tone of arterioles and venules. In this way, the cardiac and vasomotor centers indirectly affect both macrocirculation and microcirculation´s driving pressures by regulating upstream flows and pressures (Ganong, 2005).

The stimulus for activating the baroreflex is an increase in intravascular pressure. Once activated, the baroreflex will have an inhibitory effect in the cardiac and vasomotor centers. When the increase in intravascular pressure is sensed and transmitted by the reflex´s afferent arch, the information is integrated in the *medulla oblongata*. The *medulla oblongata,* in turn sends an inhibitory signal to the vasomotor center, which initiates a vasodilator-bradycardia vagal-mediated effect to counteract the increased arterial pressure (Bonanno, 2011). The opposite reaction occurs when blood pressure decreases, which leads to a decrease in baroreceptor activity (to values lower to 50-60 mmHg baroreflex activity actually ceases). In HS, it has been estimated that decreased baroreflex activity occurs when blood losses exceed 10-15% of total blood volume (Foëx, 1999). Being free from baroreflex´s restraining actions, the cardiac and vasomotor centers in the reticular activating substance of the lower pons and *medulla oblongata* increase sympathetic activity in the heart and vessels. Initially, this is manifested by an ultra-fast and neurological-mediated increase in heart rate, myocardial contractility and vasomotor tone of peripheral blood vessels. An increased secretion of catecholamines by the adrenal medulla follows next, as this gland is also a target organ of the overall sympathetic system stimulation. Experimental studies have shown

that this release of catecholamines is essential to mount an adequate response to blood loss (Baker et al., 1988).

The effectors of the sympathetic system are the catecholamines noradrenaline and adrenaline. They induce tachycardic/inotropic and vasoconstrictor actions by stimulation of β_1- and α_1-receptors in heart and blood vessels, respectively. In the heart, the main result is an increase in CO. The tachycardic and inotropic response of catecholamines is also facilitated by a concomitant decrease of acetylcholine release by the vagus nerve, because this has a bradycardic effect by interacting with muscarinic receptors.

In arterioles, catecholamine's immediate vasoconstrictor effects are an increase in systemic vascular resistance (SVR) (Ganong, 2005). This vasoconstrictor response does not occur homogenously through all arteriolar beds, with some being more affected than others. The purpose of this differential vasoconstrictor response is to lead to an effective redistribution of blood flow from the skin and connective tissue, and subsequently, from visceral organs such as gut, liver, lungs, kidney and spleen toward the more vital organs heart and brain. In this way, the body tries to preserve tissue perfusion in the organs more critically dependent on oxygen supply and "considered" more vital. This priority pattern has been engineered by Nature accordingly the organ and tissue´s capacity to be replaceable and if so, accordingly to the extent and speed that this replacement can occur. From all organs, the brain is considered the most privileged (Bonanno, 2011).The mechanisms which underlie the different response to catecholamine action manifested by the different vascular beds are not yet completely understood. It seems that they may be related with different distribution of adrenergic receptors and of iNOS. For example in Humans, but not in rats, the brain vessels have a preponderance of α_2- and β_2 instead of α_1-receptors when compared with vessels from the systemic circulation, making them insensible to the direct activity of vasoconstrictive effects of catecholamines released during HS (Nakai et al., 1986). As an opposite example we have the case of the absence of β_2-receptors in the skin/mucosae. The stimulation of β_2-receptors is associated to vasodilation. In cases of hemodynamic challenges associated to decreased blood volume, if catecholamines caused vasodilatation in the skin and mucosas, an undesirable shunt of blood could occur, diverting blood from "more vital organs" to these tissues. Another example of the differences that exist between vasculatures is the fact that brain vessels are also sensitive to pH/PCO_2 levels. This makes that the brain´s blood supply is controlled by a double mechanism: a direct mechanism, which is metabolic and based in pH and PCO_2, and an indirect, which is related with systemic blood pressure. Experimental and clinical studies have shown that in HS, the increased SVR which occurs after catecholamine´s actions mainly results from the increase in splanchnic vascular resistance. For example in dogs submitted to mild hemorrhage (resulting in tachycardia but not

hypotension), systemic blood pressure is maintained through an increase in mesenteric vascular resistance (MVR) (Foëx, 1999).

As HS becomes severer, the increase in MVR becomes more pronounced and blood flow to the GI tract, spleen, liver and kidney decreases substantially (Wang, Hauptman & Chaudry, 1990; Ba et al., 2000). In rats most affect organs by this process are the liver, kidney and GI tract (Ba et al., 2000). Interestingly the decrease in organ blood flow persists despite resuscitation, especially at the liver (Wang, Ba, Burkhardt & Chaudry, 1992; Wang, Hauptman & Chaudry, 1990). Blood perfusion to the skin is also affected, and that´s why in Humans, one of the cardinal signs of HS is the development of skin paleness.

In skeletal muscle, HS induces complex vascular changes, with increased alpha-adrenergic vasoconstrictor activity being counteracted by a concomitant increase in beta-adrenergic vasodilator activity (Haljamäe, 1984). In the early phases of HS this is manifested by a relative vasodilatation of skeletal muscle´s vasculature. The purpose of this response is to provide oxygen and nutrients to the muscle, in order that this becomes prepared to initiate the “fight or flight response”. In addition the balanced alpha and beta adrenergic activity leads to a compensatory mobilization of interstitial fluid into the vascular compartment to compensate the hypovolemia (Foëx, 1999).

Depressor reflex

When blood loss exceeds a volume more than 20% of total blood volume, the so called “depressor reflex” may occur. This consists in hypotension and bradycardia, and results from a decreased activation of the sympathetic-mediated vasoconstrictor response associated to a simultaneous increased activation of the vagus nerve (Foëx, 1999).The afferent limb of this reflex is still incompletely understood although it may be related with intense stimulation of β_2-receptors in striated muscles (Bonanno, 2011). The stimulation of these receptors induces an effect similar to when an emotional stimuli leads to a direct stimulation of the hypothalamus of the cardiovascular centers in the midbrain, inhibiting the sympathetic center and simultaneously stimulating the vagal center to the heart.

Experimental and clinical studies (Jacobsen, Søfelt, Sheikh, Warberg & Secher 1990; Jacobsen & Secher, 1992) have shown that when blood loss reaches 44% of the total blood volume, a third phase of HS may develop characterized by the presence of tachycardia and hypotension. This phase can also occur when the duration of HS is considerable (Torres et al., 2004). This phase is characterized by a renewed increase in sympathetic activity, which is however unable to maintain blood pressure and to antagonize the decrease in SVR (Angele, Schneider & Chaudry, 2008; Torres et al., 2004). Contrarily to the two previous

stages, the third stage is poorly amenable to resuscitation with blood, artificial fluids and vasopressor substances, suggesting that there is a possible state of vasomotor paralysis (Angele, Schneider & Chaudry, 2008). The underlying mechanisms for this state are incompletely understood. Proposed mechanisms include the development of adrenal insufficiency (Rushing et al., 2006) and vasopressin deficiency (Angele, Schneider & Chaudry, 2008). Microcirculatory disturbances can also play a role, as we will see in the following section.

Besides baroreflex and depressor reflexes, there are also other reflexes which are activated with HS. One of them is the chemoreflex, which is triggered by hypoxemia. Chemoreceptors which are present in the carotid and aortic bodies where baroreceptors also lie, respond to decreased PaO_2 associated to decreased CaO_2. The chemoreflex also directly stimulates the vasomotor center in the medulla oblongata (the so-called ischemic CNS response) to increase blood pressure. Thus brain hypoxia, through the stimulation of the chemoreflex, can lead to an autonomous discharge of the vasomotor center, which leads to increase blood pressure independently from the action of peripheral baroreceptors. The intense stimulation of the vasomotor center by the chemoreflex occurs at values of pressures lower than 60 mmHg and represents a last resort to increase total blood pressure and by extension, brain perfusion.

Another action of the chemoreflexes is to stimulate the medulla and pons´s respiratory centers to increase minute ventilation. This response seems to be fundamental to survival to HS, as experimental studies have demonstrated that the animals which had higher respiratory rate, lower PaCO2 and higher PaO_2, had increased survival to HS (Torres et al., 2004).

Hypoxic vasoconstriction in the lungs is another reflex which has a protective role in HS. In effect, it shuts down arterial supply in areas of the lung exposed to low FiO_2 and diverts blood to better ventilated areas. Finally atria and pulmonary artery have low-pressure baroreceptors responding to an increase of pressure with an increase of heart rate and to decreased vasopressin secretion by the hypothalamus; other atrial receptors directly stimulate the sinus node to increase rate on increase of pressure. These reflexes turn out useful on readjustments after volume overload in normal or pathological situations.

Importantly, other areas in the brain such as the hypothalamus and the cerebral cortex can also affect the vasomotor center´s response and the baseline vasoconstrictor tone and these may also play a role in the compensation to HS.

2.9. HS and microcirculation

Besides the changes described in global and regional hemodynamics, HS also decreases microvascular blood flow (Wang, Hauptman & Chaudry, 1990). Microvascular dysfunction is currently considered one of the most important factors for the development of MOF following shock states (Doerschug, Delsing, Schimdt & Haynes, 2007; Garrison, Spain, Wilson, Keelen & Harris, 1998).

Microvascular dysfunction can in part be due to development of a state of "arteriolar hyposensitivity", which is manifested by a progressive arteriolar vasodilatation and a decreased responsiveness of the microcirculation to alpha-agonists. Interestingly, it seems that microvascular vasodilatation is just the initial step of a state of vasomotor dysfunction, which eventually spreads to the regional and systemic vessels, leading to a decrease in SVR. This state of “vasomotor paralysis” is characteristic of decompensated HS and is associated to a poor prognosis. Several reasons have been advanced to explain the development of this “arteriolar hyposensitivity” and “vasomotor paralysis”. One of them states that this is related with the sensitivity of the vascular contractile apparatus to calcium. Calcium influxes, either from extracellular fluid or from the ER, are determinant for the final pathway to vascular contraction (Bonanno, 2011). It is known that vascular reactivity and calcium sensitivity are increased in early shock (beginning immediately up to 30 min after the initiation of HS) but that both decrease as shock progresses (after 1 to 2 hours post shock onset). The changes in vascular reactivity and calcium sensitivity are related with noradrenaline and Ca^{2+} concentrations (Bonanno, 2011). The decrease in vascular reactivity seems to result from a desensitization of the vascular smooth muscle cell apparatus to Ca^{2+}, which in turn may derive from changes in Rho-kinase activity. In factthis enzyme seems to regulates vascular reactivity by regulating Ca^{2+} sensitivity (Li, Liu, Xu, Yang & Ming, 2006).

Increased levels of NO have also been suggested as a cause of the vasomotor paralysis. HS upregulates calcium-dependent constitutive nitric oxide synthetase (cNOS) and endothelial nitric oxide synthetase (eNOS) and induces the expression of iNOS. This culminates in increased production of NO which in turn can cause vascular hyporeactivity (Thiemerman, Szabó, Mitchell & Vane, 1993). NO has several physiological functions in many processes including the maintenance of microvascular homeostasis by regulating arteriolar tone, RBC and leukocyte deformability, leukocyte-endothelial adhesion in mesenteric and skeletal muscle postcapillary venules, platelet adhesion and aggregation to endothelial cells, blood volume and mitochondrial respiration (Hauser, Matejovic & Radermacher, 2008; Bateman, Sharpe & Ellis, 2003). In moderate amounts, increased NO production is believed to have

beneficial effects in microcirculation (Bonanno, 2011). In the presence of stimuli that stimulate vasodilation such as hypoxia and acidosis, endothelial cells of arterioles are stimulated to produce NO. NO than diffuses to the smooth muscle cell, where through a guanylate cyclase/cGMP-mediated reaction, it induces relaxation of the smooth muscle and arteriole dilation. By this way NO production opposes sympathetic or chemically mediated vasoconstriction. In addition. it prevents leukocyte-endothelial cell adhesion, scavenges ROS and reduces oxygen consumption (Hauser, Matejovic & Radermacher, 2008; Bateman, Sharpe & Ellis, 2003). However, when the production of NO becomes exaggerated, deleterious consequences develop in the host. At the vascular level, excessive NO production leads to an excessive decrease in vascular tone caused by decreased vascular sensitivity to catecholamines and abnormal vascular reactivity (Chen, Li, Shih, Liao & Wu, 2010). Overproduction of NO is also associated to a decrease in blood pressure and SVR and it has been considered the main cause of refractory hypotension observed in sepsis. Other deleterious effects of NO overproduction include an inhibition of AVP release (Giusti-Paiva, De Castro, Antunes-Rodrigues & Carnio, 2002), increased vascular permeability in the intestine, heart, liver and kidney (Bonanno, 2011), impaired microvascular reactivity, reduced RBC deformability and decreased functional capillary density (Hauser, Matejovic & Radermacher, 2008; Bateman, Sharpe & Ellis, 2003).

In recent years, the release of endothelin by the damaged endothelium has also been incriminated in HS-associated vasomotor paralysis (Sharma et al., 2002). Other potential etiologies which have been proposed include increased opening of ATP-sensitive K^+ channels due to decreased intracellular levels of ATP and intracellular acidosis (Horiuchi, Dietrich, Hongo, Goto & Dacey, 2002) and AVP and corticosteroid deficiency. AVP and corticosteroid deficiency will be discussed later, in the section of HS-associated endocrine changes.

In addition to refractory microvascular vasodilatation, HS is associated to other microcirculation disturbances, including the phenomenons of capillary "no-flow" with prolonged ischemia or "no-reflow" with reperfusion. Intravital microscopic studies performed in animals submitted to HS have shown that in the early post-HS period, microvascular blood flow in skeletal muscle initially becomes intermittent, and as time progresses, it stops completely (Haljamäe, 1984). Interestingly, the same studies demonstrated that after resuscitation, only 30% to 50% of the capillaries which were perfused before HS onset, regain its blood flow and that this is achieved in an heterogeneous manner (Haljamäe, 1984). Besides, HS-associatedmicrocirculatory changes are known to persist for several days after shock onset (Tachon et al., 2014).

What causes microcirculatory dysfunction in critical illnesses such as HS is still incompletely understood and most of our knowledge in this matter cames from clinical and experimental studies concerning sepsis. Sepsis induces many types of structural and functional changes in the microcirculation,including endothelial dysfunction (manifested by a deficient or impaired reactivity); vasoparalysis (which can become irreversible) and oxygen maldistribution and capillary stop flow (resulting in dysoxic-hypoxia) (Vallet, 2003). Sepsis-induced endothelial dysfunction may be mild or severe. In the former case, sepsis induces a form of light structural damage which is associated to decreased endothelial-dependent vascular relaxation(Bonanno, 2011). This condition known as “endothelial stunning”, is believed to be due to decreased NO formation and release and it is usually reversible, although it can persist for several days. In contrast, when the endothelial damage is severer, a form of refractory vasomotor paralysis which is irreversible occurs. The causes of sepsis-induced irreversible endothelial dysfunction are still poorly understood. Both increased NO production and endotoxin-mediated damage have been suggested. In fact, endotoxin has been shown to impair endothelial cell uptake of O_2 (Motterlini, Kerger, Green, Winslow & Intaglietta, 1998) and to inhibit NO-induced microvascular dilation (Engelberger et al., 2011). Capillary stop flow maybe caused by microvascular stasis, fibrin deposition, altered RBC deformability, altered platelet´s aggregation or adhesion properties, increased leukocyte adhesion, reduced leukocyte deformability, endothelial swelling and microthrombi formation. The increased heterogeneous expression of iNOS in different vascular beds which occurs with sepsis can also play a role in the pathological shunting of flow observed in this condition. In fact, in sepsis, areas which are lacking iNOS have less NO-induced vasodilation and become underperfused where the ones with increased NO production suffer from the pathological effects of its overproduction.

The hallmark of microcirculatory dysfunction in sepsis is the development of dysoxia, which consists in the discrepancy between DO_2 and oxygen extraction. Dysoxia is believed to be initially caused by maldistribution of blood flow. This in turn presents itself as a phenomenon of “spatial and temporal heterogeneity of capillary flow”. The existence of maldistribution of blood flow has been documented by microscopy in tissues of human and animal septic patients. At microscopic observation, areas with both reduced and increased capillary density coexist. Capillary blood flow becomes also heterogeneous , with some capillaries and venules having sluggish flow or no flow at all and others, in contrast, presenting normal and even abnormally high blood flow. The loss in tissue perfusion associated with the absence of flow in some of these capillaries is accompanied (and correlated) with impaired O_2ER and increased SVO_2. In contrast, in the adjacent areas which have normal capillary blood flow, O_2ER is increased and SVO_2 decreased. The loss of capillary blood flow also contributes to an extended presence of proinflammatory mediators in microcirculation and tissues,

potentiating its effects. In the end, heterogenous capillary blood flow leads to impaired SVO_2, local tissue hypoxia, necrosis, inflammation and ultimately organ dysfunction.

Microcirculatory dysfunction has been considerably less studied in HS than in sepsi. These studies mostly consist in experimental studies (Van Genderen et al., 2014; Maier et al., 2009) although there are also clinical studies in dogs (Peruski et al., 2011) and Humans (Tachon et al., 2014). HS-induced macrocirculatory disturbances resemble those reported in sepsis (Tachon et al., 2014). They can last more than 72 hours after restoration of macrocirculation disturbances (Tachon et al., 2014). They also seem to be independent with catecholamine levels and/or substrate exhaustion (Baker et al., 1988; Perbeck & Hedqvist, 1982). Most of its underlying mechanisms remain largely understood. A large observational study in Humans is currently underway to address the effects of traumatic injury, HS and resuscitation on the microcirculation which possibly will provide us with more information regarding this subject (Hutchings et al., 2016).

2.10. Endocrine response to HS

HS induces several changes in the endocrine milieu of the host. In 1932, Cuthberson provided one of the first detailed descriptions of the metabolic response to critical injury, in four patients with lower limb injuries. This first description included a quantification and evolution of these changes across time. Cuthberson (Cuthebertson, 1982) and other authors (Moore, 1953; Selye, 1952), based in experimental and clinical evidence, introduce the first teleological concepts of the metabolic response to injury. Accordingly to these, the metabolic response to injury involves two subsequent phases. The first phase is called the ebb-phase and the second the flow phase. The ebb phase lasts 24 to 72 hours, is characterized by hypometabolism and has the main goal of stabilization of the body´s clinical condition after injury to preserve the internal milieu. The flow phase which lasts normally two weeks (it may last up to eight weeks or longer in severer cases) is characterized by hypermetabolism and is when convalescence occurs . Both phases result from an integrated response of the nervous, endocrine, and metabolic systems which “sacrifice” normal body´s functions to achieve specific survival objectives (protection, stabilization and adaptation). Systemic changes (such as tissue catabolism) become devoted to caloric needs and local growth (wound repair) with the ultimate goal being the body´s survival.

2.10.1. The stress response

HS induces what it is called a stress response (Figure 7). Seyle was the first to introduce the concept of stress response or general adaptation syndrome. Thi is defined as a syndrome produced by “diverse nocuous agents”, consisted by a sequence of three stages: alarm reaction, resistance, and if the stressor does not relent, exhaustion (Seyle, 1952).

Stress on the other hand is the resource-intensive process of mounting allostatic responses to challenges that occur in the external or internal environment. A *stressor* is any event that elicits a *stress response*. There are several possible stressors. These include a physical or social event, an invading microorganism, the presence of tissue trauma or for the purpose of this discussion, the development of HS.Stressors can be characterized by intensity, duration and frequency and their impact is in function of the magnitude of the response it elicits.

Allostasis consists in the adaptive process that develops with the aim to return to homeostasis, once the initial equilibrium has been challenged. The stress response is a perfect example of allostasis, because it mobilizes internal resources to meet the challenge that a stressor represents. The cost to the body, or burden, of allostatic adjustment, is called *allostatic load*.

Accordingly to DeKloet and Derijk, 2004, the stress response has two states. The first state, also called the fast state, consists in the immediate arousal in response to the stressor and enables adaptive behaviors. The second state, the slow state, follows the fast state and consists in a process that promotes recovery, behavioral adaptation and return to normalcy. Others have designated these states as *defensive arousal* and *recovery* for the first and second state respectively (Chapman, Tuckett & Song, 2008). Interestingly these terms seem to overlap with ebb and flow phase proposed by Cuthbertson. In fact they may represent different terms for the same phenomenon.

The major mechanisms of the stress response in the defensive arousal stage include the activation of the *locus coeruleus* noradrenergic system, of the HPA axis based in the hypothalamic periventricular nucleus (PVN), and of the sympathoadrenomedullary axis.

The peripheral effectors of these mechanisms are the autonomic nervous system and the hormones produced by the sympathoadrenomedullary and HPA axis. The sympathoadrenomedullary circulating hormones include the catecholamines epinephrine and norepinephrine together with the sympathetic co-transmitter neuropeptide Y, all of which originate in the chromaffin cells of the adrenal medulla. The hormonal effectors of the HPA axis include peptides derived from pro-opiomelanocortin (POMC) at the anterior pituitary. The POMC-related family of anterior pituitary hormones includes ACTH, β-lipotropin, β-melanocyte stimulating hormone (α-MSH) and β-endorphin. ACTH will lead to the release of cortisol (CS in rats) by the adrenal cortex.

The overall goal of these hormonal changes is to increase catabolism, mobilize substrates to provide energy sources and retain salt and water to maintain blood volume and cardiovascular homeostasis. In evolutionary terms this make senses, because it permits the animals to survive and sustain themselves without food until injuries have healed and repair has taken place (Desborough, 2000). Studies in the last decades have shown that the changes in the endocrine systems are tightly coordinated with the actions of the neurological and immune systems and that the latter serve the same purpose. In the following sections, the main hormonal changes induced by injury in general and HS in particular will be discussed.

Figure 7: hormonal changes following the advent of a stressor

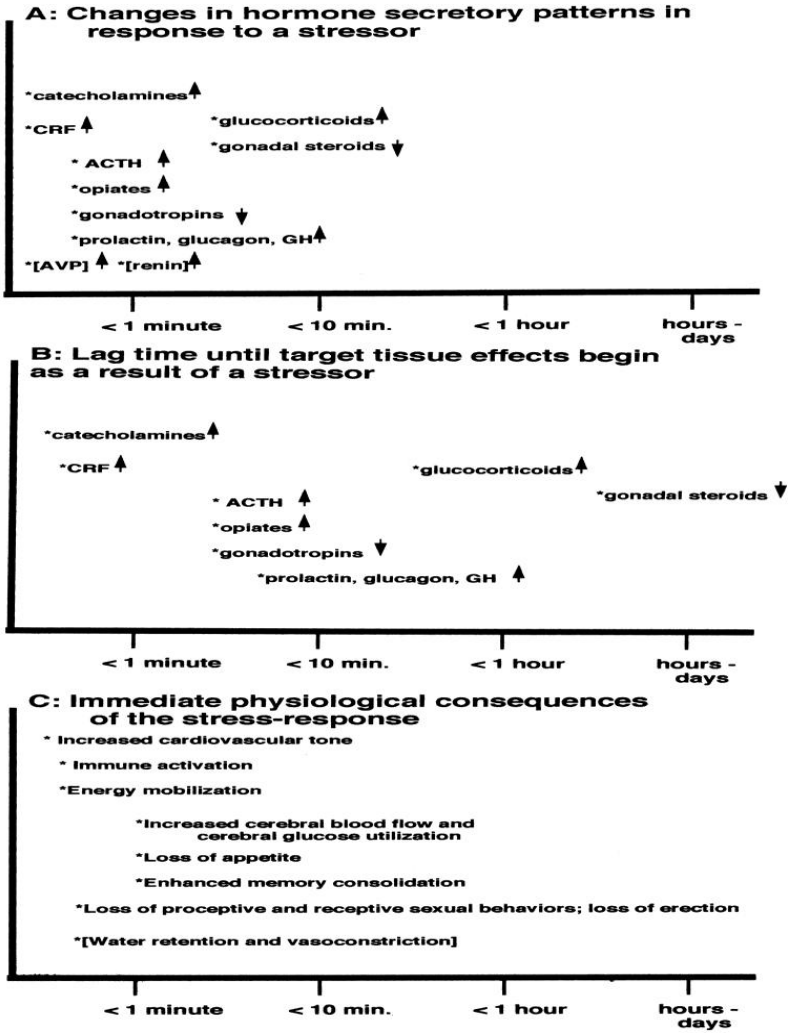

Figure 7: Schematic overview of the typical endocrine stress response. **A**, The time course of changes in hormone-secretory patterns in response to a stressor. **B**, The lag time until target tissue begin as a result of a stressor. **C**, Immediate physiological consequences of the stress response. *Asterisks* represent approximately where in the time line, the first effects of a particular hormone began to be felt (A and B) or when n the time line the physiological consequence is initiated (C). There is no formal y axis-hormones or consequences are simply spaced vertically to facilitate reading. Adapted from Sapolsky, Romero & Munck, 2000.

2.10.2. Sympathoadrenal response

As described before, HS induces activation of the sympathetic nervous system, which results in the secretion of catecholamines by the adrenal and norepinephrine by presynaptic nerve endings. In rats, HS results in an immediate and marked elevation of circulating epinephrine and norepinephrine levels (10- and 2-fold, respectively) (Molina & Abumrad, 1999). Although the latter is a neurotransmitter, there is always a small amount of norepinephrine which spills into the circulation. Besides its cardiovascular effects, adrenaline and noradrenaline increase glycogenolysis (especially in the ebb phase), gluconeogenesis and lipolysis. Increased catecholamine levels are also one of the causes of the insulin resistance which accompanies trauma and HS (Maier, 2005).

It is also known that sympathetic nervous system´s activation has effects in the immune system. Sympathetic axon terminals which innervate lymphoid tissues release adrenaline, noradrenaline and neuropeptide Y, which affect lymphocyte, macrophage and other immune cell´s function (Scanzano & Cosentino, 2015; Miksa, Wu, Zhou & Wang, 2005). The release of catecholamines in systemic circulation exerts a similar effect. Catecholamine secretion modulates all aspects of immune responses and can alter lymphocyte proliferation, cell trafficking, antibody secretion, and cytokine production (Elenkov & Chrousos, 2002). Neuropeptide Y stimulates lymphocyte proliferation (von Horsten, 1998) and enhances leukocyte function (Sung, Arleth & Feuerstein, 1991). More specifically in HS, studies have demonstrated that the catecholamine response has significant immune effects (Molina, 2001; Molina & Abumrad, 1999) especially by affectingtissue cytokine and β-endorphine circulating levels (Molina, 2001; Molina & Abumrad, 1999). Some authors actually believe that this release of catecholamines is pivotal for resolving the local inflammatory response (Molina, 2005). Studies are ongoing to better clarify what is the clinical significance of sympathetic nervous system effects in the immune response to HS.

2.10.3. Hypothalamic–pituitary-axis (HPA)

The primary site of the CNS that governs the HPA axis is the hypothalamic PVN. The PVN is divided functionally and phenotypically in two major neurosecretory subdivisions: the magnocellular (mPVN) and the parvocellular groups (pPVN). The mPVN together with the supraoptic nucleus constitute the magnocellular neurons, which are the main responsible for vasopressin (AVP) and oxytocin production and which are released into systemic circulation by the terminal portions of the neurons located in the posterior pituitary. The more medial pPVN constitute the major source of hypophysiotrofic Corticotrophin-releasing hormone(CRH) neurons. CRH is the major physiologic regulator of ACTH secretion.

Consistent with the several possible stimuli that can activate the HPA axis, the pPVN is connected to several other areas of the CNS. These include projections from other nuclei in the hypothalamus (e.g. medial preoptic anterior hypothalamus), from the *Nucleus Tractus Solitarius*, medullary catecholaminergic cell groups, *organum vascularis* of the lamina terminalis, the subfornical organ and the blood brain barrier (Turnbull & Rivier, 1999). Vasopressin is also known to stimulate ACTH secretion (Peetters et al., 2014). The CRH-producing neurons project into the external zone of the median eminence (ME) and release CRH into a specialized capillary network. From this capillary network arise hypophyseal portal vessels which supply the adenohypophysis. In here CRH, stimulates the production of the ACTH-precursor, POMC and the secretion of ACTH, β-endorphine and α-melanocyte stimulating factor through its ligation to a specific receptor named CRF-R1 (Bonfiglio et al., 2011). The classical HPA axis pathway is completed by ACTH-induced secretion of cortisol, in Humans and CS, in rats and mouse, by *zona fasciculata* of the adrenal gland (Smith & Vale, 2006). In a classical feedback system, glucocorticoid secretion inhibits the synthesis and secretion of CRH in the hypothalamus and of ACTH and other POMC-derived peptides in the pituitary (Turnbull& Rivier, 1999).

Following HS, the HPA is rapidly activated, leading to increased production and secretion of cortisol, in Humans, and CS in rats (Gundersen et al., 2003; Machuganska & Zaharieva, 1985). In rats, the HPA response is so fast that a peak in ACTH and CS levels has been reported to occur in 9 and 18 minutes post-HS respectively (Thrivikraman & Plotsky, 1993). A similar pattern has been reported in an experimental model of HS by decreasing lower body pressure in Humans (Pitts, Preston, Jaeckle, Meller & Kathol, 1990).

Although howHS leads to HPA activation is still incompletely understood, it is known that this can occur through different stimulus from different origins . These will act in combination at different levels. One of these stimuli is hypotension, which is transmitted from cardiovascular receptors to the hypothalamus through ascending neural pathways (Gann, 1979). Hypotension seems to be an important factor to increase ACTH release in HS (Turnbull & Rivier, 1999) as hemorrhage without hypotension has been associated to increases of vasopressin but not of ACTH circulating levels (Buller, Smith & Day, 1999). Increased levels of angiotensin II following hemorrhage have the same action but act in the median eminence (DeMaria, Lilly & Gann, 1987; Gann, 1979).

Another source of HPA activation is the stimulation of nociceptive receptors after injury (Bereiter, Benetti & Thrivikraman, 1990). All these stimuli are integrated in the *Nucleus Tractus Solitarius* in the medulla oblongata, which is pivotal for the occurrence of ACTH release (Darlington, Shinsako & Dallman, 1986).

Activation of the HPA axis by cytokines

Immune system activation can also stimulate HPA axis activity, in particular through cytokines. In this regard, the first cytokine which demonstrated stimulatory effects in the HPA axis was IL1. It is now known that TNF-α and several cytokines from the gp130 cytokine family, in particular IL6 and Leukemia-Inhibitory factor share IL1´s effects (John & Buckingham, 2003). Nevertheless they are less potent and effective than IL1. Small increases in body temperature occur in response to IL1, IL6 and TNF-α alpha, but these changes can be prevented by COX inhibitors and do not appear to be related with HPA-activation. In addition, and although it is known that the rapid HPA-activating effects of IL1 are impaired by COX inhibitors, the prolonged HPA activation which follows intraperitoneal injections of IL1 is not. This indicates that IL1 actvates the HPA axis through several mechanisms.

Cytokines can stimulate the HPA axis by acting directly at the hypothalamus, pituitary gland and adrenal. At the hypothalamus pro-inflammatory cytokines increase the secretion of CRH by several mechanisms (Mulla & Buckingham, 1999). One is through direct induction of CRH release by hypothalamic neurons. In this case, cytokines transported by the systemic circulation contact directly with hypothalamic neurons through specific receptors, after entering the brain through the fenestrated capillary endothelium of the circumventricular organs, in sites where the blood-brain-barrier permeability is increased (Turnbull & Rivier, 1999).They can also stimulate brain perivascular cells to produce inflammatory mediators such as eicosanoids or other cytokines, which then act on hypothalamic neurons to induce CRH release (John & Buckingham, 2003). The latter mechanism explains the inhibition of HPA-activation induced by IL1 and TNF-α with the use of COX inhibitors (Turnbull& Rivier, 1999; Dunn, 2000). At the pituitary levels, cytokines of the gp130 family such as IL6 can also increase directly the release of ACTH by corticotrophs by ligation to the IL6 receptor (IL6R) (John & Buckingham, 2003). At adrenal level cytokines such as IL1 and IL6 have also been shown to increase the release of glucocorticosteroids. This occurs by both direct actions and by increasing the steroidogenic effects of ACTH. Cytokines can also activate the HPA axis through indirect mechanisms. One of them is by stimulating directly local sensory afferent fibers which than carry the sensory information to the *Nucleus Tractus Solitarius* and from here to the hypothalamus (Layé et al., 1995). The can also activate the HPA axis by inducing pathological changes such as hypotension (Turnbull & Rivier, 1999).In addition, a local production of cytokines occurs at several levels of the HPA axis, influencing its activity through paracrine and autocrine mechanisms (John & Buckingham, 2003). Finally

The different hormones of the HPA axis

Recent studies have increasingly shown that many of the hormones involved in the HPA axis, including CRH, ACTH, α-MSH and β-endorphin, have more functions than those which have been initially found. These subjects will be discussed briefly in the following sections.

CRH

In addition to its role in stimulating ACTH release, CRH occurs diffusely in the brain where it can act as a neurotransmitter (Sapolsky, Romero & Munck, 2000). Many behavioral aspects of the integrated stress response such as locomotor activity, food intake, sexual behavior, sleep, arousal, anxiety, learning, and memory formation are attributed to neuronal CRH projections including neocortical, limbic, and brainstem structures, especially anxiety-like behavior (Bonfiglio et al., 2011).Importantly it can increase sympathetic arousal, thus becoming a pivotal substance that establishes a link between the HPA axis and the SNS.

ACTH

ACTH is peptide belonging to the melanocortin family (Bitto et al., 2011). It is now known that ACTH has beneficial effects in HS that go beyond its effects in increasing glucocorticoid production. This has been demonstrated in both experimental models and clinical studies in patients with HS and where ACTH has been shown to support blood pressure and to increase survival (Bazzani, Balugani, Bertolini & Guarini, 1993; Bertolini et al., 1987). The mechanisms in how ACTH leads to these beneficial actions are yet incompletely understood. It is known in rats that ACTH administration reduces intra-cellular adhesion molecule expression, leukocyte infiltration and vascular dysfunction (Squadrito et al., 1999). Part of these effects can result from an ACTH-mediated TNF-α inhibition (Altavilla et al., 1998) and from decreasing iNOS synthetase activity (Bazzani, Bertolini & Guarini, 1997). More recently it was demonstrated that ACTH-(1–24), through the activation of CNS melanocortin 4 (MC4) receptors, triggers the activation of the vagus nerve-mediated brain cholinergic anti-inflammatory pathway. The latter, through involvement of brain muscarinic and, as main final step, of liver nicotinic receptors leads to the suppression of NF-kB and inflammatory cascade activation, and reversal of the shock condition (Guarini et al., 2004).
Studies have also shown that other peptides belonging to the melanocortin system, such as α-MSH also possess beneficial effects, which are also probably mediated by the activation of efferent vagal fibers and which also involve the ligation to the MC4 (Bitto et al., 2011). Beneficial effects of α-MSH that have been demonstrated from several models include

inhibition of nuclear NF-kB activation, reduction of pro-inflammatory cytokine and adhesion molecule production, up-regulation of IL10 and increased survival (Catania et al., 2010; Ottani et al., 2009; Getting, 2006; Guarini et al., 2004; Squadrito et al., 1999; Altavilla et al., 1998). In addition reduced concentrations of circulating α-MSH have been detected in critically ill patients with a worse outcome, including trauma patients (Todd et al., 2009), patients with septic shock (Catania et al., 2000) and brain injury (Magnoni et al., 2003). This suggests that melanocortin peptides have a physiological and mainly anti- inflammatory role.

Glucocorticoids

As described before, glucocorticoid´s levels increase in the systemic circulation in minutes after the stressor initiates the stress response (Sapolsky, Romero & Munck, 2000). The bulk of glucocorticoid actions are exerted at genomic level, through GCR-mediated changes in gene transcription (Distelhorst, 2002). Consequently, most of its actions are not exerted until about one hour after the onset of the stressor. In general, the actions of glucocorticoids can be described as permissive, suppressive, stimulating and preparative. The permissive actions of glucocorticoids are present before the stressor and prime the body´s defense mechanisms to react to stress. The suppressive actions occur in an hour or more after the stress response begins and have mainly the goal to restrain the latter (Figure 8). The stimulating effects also occur in an hour or more after the stressor, and contrary to suppressive actions, they potentiate several aspects of the stress response. In fact in order for the stress response to be successful s both glucocorticoid stimulating and supressive actions must be present. Finally, the preparative actions occur much later than the immediate response to the stressor and do not affect it directly. By contrary, they help to prepare the organism for future threats. Preparative actionscan be both mediating or suppressive. In this section, we will discuss several general actions of glucocorticoids which are more relevant for HS, especially thepermissive, suppressive or stimulatory actions. For preparative actions, the reader is advised to look elsewhere in the literature (Sapolsky, Romero & Munck, 2000).
Glucocorticoids act in many systems in the body through genomic and non-genomic mechanisms. Genomic mechanisms involve the ligation of glucocorticoids to the GCRalthough they can also interact with mineralocorticoid receptors. Almost every cell in the body have GCR although their number varies accordingly to cell type. The regulation of glucocorticoid´s expression, activity, half-life and binding capacity is modulated by receptor phosphorylation, which is upregulated by binding. This can play a significant role in the tissue sensitivity to glucocorticoids activity, especially in conditions of prolonged stress.
The actions of glucocorticoids are regulated by the activity of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HDS), which exists in two isoforms: 11β-HDS1 and 11β-HDS2. 11β-

HDS2 exists mainly in the kidney and in Humans, it catalyzes the almost irreversible inactivation of cortisol into corticosterone. This reaction protects the nonspecific mineralocorticoid receptor present in those cells from being activated by cortisol. In this manner in these cells the mineralocorticoid receptor is aldosterone-selective (Edwards, 2012). 11β-HDS1 catalyzes both the oxidizing (inactivating) and reducing (activation) reactions. 11β-HDS1´s main location is the liver where it exerts an important role for inducing the glucocorticoid´s metabolic response to stress.

The way how glucocorticoids influence gene activity through their ligation to GCRs is reasonably understood. Unligated GCRs are predominantly cytoplasmatic although they probably cycle between the cytoplasm and nucleus. They form large heterocomplexes with HSP90 and other HSPs. Once glucocorticoids ligate with its specific receptors, they become activated, and the heterocomplex dissociates, releasing the complex glucocorticoid-GCR. This becomes highly phosphorylated and moves into the nucleus. Once in the nucleus, the glucocorticoid-GCR ligates to short palindromic sequences of nucleotides in their gene promoter region. These sequences are called glucocorticoid response elements (GREs) and once the ligation takes place, gene transcription is initiated. In the case of negative GREs (nGREs), the binding of the glucocorticoid-GCR repress its expression, by displacing or interfering with positively acting factors. The complex glucocorticoid-GCR can also interfere with gene transcription without ligation to GREs and nGREs, in a process called transcriptional “cross-talk” via factor tethering. In this process, glucocorticoids ligate directly to other transcription factors through its binding site. This process is inhibitory in most cases although it can also be synergic (Sapolsky, Romero & Munck, 2000).

Glucocorticoids can act also through non-genomic mechanisms. It is believed that non-genomic mechanisms are the most likely mechanism of glucocorticoid´s faster responses (seconds to minutes), including the negative glucocorticoid feedback mechanism to inhibit stress-induced ACTH secretion. The way how glucocorticoids operate through non-genomic actions is still incompletely understood. Recent evidence suggests that these are mediated by the intracellular protein annexin-1, although the role of endogenous cannabinoids in this regard has also been suggested (Ginsberg, Pecoraro, Warne, Horneman & Dallman, 2010).

Effects of glucocorticoids in HS

Some of most relevant actions of glucocorticoids during HS are related with its effects in metabolism and in the cardiovascular, neurologic and immune systems. These will be briefly described below:

Metabolic effects: the metabolic effects of glucocorticoids in HS serve mainly to maintain the high levels of blood glucose which were initially induced by the early release of

catecholamines, glucagon and growth hormone. Glucocorticoids produce these effects by several mechanisms including: appetite stimulation; induction of glycogenolysis; stimulation of hepatic gluconeogenesis and glycogen deposition; inhibition of peripheral glucose transport and utilization; mobilization of lipids through stimulation of lipolysis in fat cells; mobilization of amino acids through inhibition of protein synthesis and stimulation of proteolysis in several muscle types. In this regard the metabolic actions of glucocorticoids are both permissive and stimulatory.

Cardiovascular effects: the effects of glucocorticoids in the cardiovascular stress response are well understood. Glucocorticoids increase blood pressure and CO. Although glucocorticoids have a positive inotropic action, their cardiovascular effects are mostly permissive, by facilitating the action of catecholamines and other vasoconstrictors. This permissive action is exerted by several ways. Glucocorticoids induce phenylalanine-N-methyltransferase, the rate-limiting enzyme of adrenaline synthesis; prolong catecholamine actions in the neuromuscular junction by inhibiting catecholamine reuptake and metabolizing enzymes; increase the binding capacity and affinity of β-adrenergic receptors to cathecolamines in arterial smooth muscle cell´s, receptor-G protein coupling and catecholamine-induced cAMP synthesis; increase adrenergic receptor mRNA levels and diminish prostaglandin synthesis (Sapolsky, Romero & Munck, 2000). When the stressor is blood loss, glucocorticoids have also another physiological role. Hemorrhage induces the release of AVP and renin, to increase water restriction and vasoconstriction. Glucocorticoids have been shown to inhibit AVP release, to increase glomerular filtration rate and to induce the secretion and efficacy of atrial natriuretic peptide (ANP) (Hayamizu, Kanda, Ohmori, Murata & Seo, 1994). Theoretically these actions would lead to a decrease in blood pressure. How can the antagonism of AVP can be beneficial for HS? The answer was provided by several studies (Darlington et al., 1990; Darlington, Keil & Dallman, 1989). These revealed that when adrenalectomised rats were submitted to HS and this proved uniformly fatal, one reason for mortality was the abnormally high levels of AVP. What occurred was that becauseadrenalectomised rats did not have glucocorticoids in adequate concentrations to oppose the high AVP levels. Being unrestrained, these led to a severe vasoconstriction of the hepatic, gastrointestinal and coronary circulation with subsequent severe organ ischemia and subsequent death.

Immunological effects: Glucocorticoids can have both immunosuppressive and immuno-enhancing actions in the immune system. The most classically and known effects are the immunosuppressive effects. They inhibit the synthesis, release and/or efficacy of cytokines and other mediators involved in immune and inflammatory reactions. Mediators affected by glucocorticoids include IL1, IL2, IL3, IL4, IL5, IL6, IL12, GM-CSF, IFN-γ, TNF-α, histamine, bradykinin, eicosanoids, NO, collagenase, elastase and plasminogen activator. They inhibit

the expression of the inducible form of COX-2, the induction of intra-cellular adhesion molecule expression, antigen presentation, expression of major histocompatibility complex (MHC) type II and activation and proliferation of T and B cells. Working together with increased catecholamines levels, glucocorticoids also shift the immune response from Th1 to Th2, increase Treg activity (Marik & Flemmer, 2012) and increase IL10 and TGF-β levels (Ng, Li, Pavlakis, Ozato & Kino, 2013). Glucocorticoids´s immune suppressive effects are in part due to interference in NF-κB activity. This interference may occur by several mechanisms including induced synthesis of I-κBα and I-κBβ and decreased binding of NF-κB (Distelhorst, 2002). They also affect the AP-1 pathway and in the case of IL1, inhibit IL1 transcription, translation and secretion, by destabilizing its mRNA (Sapolsky, Romero & Munck, 2000). Glucocorticoids´s immune-enhancing effects include stimulation of immunoglobulin production by B-cells and enhancement of T-cell responses (Sapolsky, Romero & Munck, 2000). They also augment the hepatic acute–phase response by increasing the sensitivity to inflammatory mediators (Jensen & Whitehead, 1998), increase leukocyte mobilization to the sites of injury (Dhabhar & McEwen, 1997), increase cellular expression of receptors for several pro-inflammatory cytokines including IL1, IL2, IL4, IL6, IFN-γ and GM-CSF, stimulate monocyte phagocytosis (van der Goes, Hoekstra, van den Berg & Dijkstra, 2000), macrophage activation and delay neutrophil apoptosis (Yeager, Pioli & Guyre, 2011).

Glucocorticoid´s enhancement of immune function seems to be a permissive action, permitting the normal immune response at the onset of the stressor. The immuno-enhancing effects of glucocorticoids occur at doses lower than those which induce immunosuppression, and more specifically at glucocorticoid´s basal levels (Edwards, 2012) (Figure 8). These permissive effects result mainly from the stimulation of mineralocorticoid receptors, although they can also be produced by stimulation of the GCRs. Examples of the latter is the induction of receptors for IL6 (Snyers, De Wit & Content, 1990), α1 (Sakue & Hoffman, 1991), β2 (Collins, Caron & Lefkwotiz, 1988) and angiotensin II (Sato et al., 1994). At the genomic level, permissive actions are associated to increased levels of mediator receptors, mainly by gene transactivation (Sapolsky, Romero & Munck, 2000). Permissive immuno-enhancing glucocorticoid's activity are also dose-dependent. However even at high doses the permissive actions of glucocorticoids are obliterated by the immunosuppressive effects. Glucocorticoids immunosuppressive effects occur at stress doses of glucocorticoids (which are always higher than permissive doses), and are also dose-dependent (Edwards, 2012). Immunosuppressive activity is mainly caused by the stimulation of GCRs and results from gene transrepression (Sapolsky, Romero & Munck, 2000). Ample evidence exists to confirm that without the stress (immunosuppressive) doses of glucocorticoids, the outcome after a severe stressor is poor. For example adrenalectomy has been found to significantly increase

fever and mortality by sepsis (Coelho, Souza & Pela, 1992; Nakano, Suzuki & Oh, 1987). In this context, the increased mortality seems to derive from increased tissue and systemic levels of TNF-α (Zawacki , Hunt, Gamelli & Filkins, 1997). Increased TNF-α levels have also been demonstrated in adrenalectomised rats submitted to experimental HS (Yamashita & Yamashita, 2001). In addition, the administration of TNF-α and IL1 at doses normally tolerated by intact animals is uniformly fatal in adrenalectomised animals (Bertini, Bianchi & Ghezzi, 1988). A limitation of experimental studies which use adrenalectomised animals is that the pathological consequences of adrenalectomy can be attributed to both the lack of glucocorticosteroids or catecholamines. Nevertheless there is abundant evidence from other models that indicate that it is the lack of glucocorticoids which is responsible for most morbidity and mortality. For example robust cytokine responses to stress are observed in animals treated with glucocorticoids receptor antagonists or with the glucocorticoid synthesis inhibitor metyrapone (Michel et al., 2007; Nguyen et al., 1998; Fleshner, Brennan, Nguyen, Watkins & Maier, 1996). In addition, the worse prognosis associated with adrenalectomy in HS is corrected by supplementation with exogenous steroids (Hinshaw, Beller & Chang, 1985).

So, one may ask, that when an animal suffers from HS, which glucocorticoid effects are most important? Should it be the permissive, the immuno-enhancing or by contrary, the immunosuppressive? The answer is straightforward: they all are, because they all are essential for the normal stress response. In the commonly accepted view, glucocorticoids at basal levels, by acting in a permissive way, help to mediate the first wave of immune responses to stress. This consists mostlyin immune activation. Subsequently, this response is followed by the imunosupressive effects of glucocorticoids doses (figure 8). The immunosupressive effects have several purposes. The first is to limit inflammation to where it mostly needed (e.g. the site of injury). This is achieved because the increase in glucocorticoid´s systemic levels induces a systemic state of relative immunosuppression, which prevents that cytokine levels become excessive and thus toxic. In this manner cytokine-induced tissue damage is prevented (Lowry, Takeuchi, Cremisi & Konieczny, 1993).Second, glucocorticoid-related immunosuppression may serve as a protective mechanism against the development of auto-immune disease (Besedovsky & del Rey, 1991). because the latter results from the fact that glucocorticoids are more effective in targeting lymphocytes which are less active or that produce antibodies with lower affinities for the antigen (Iseki, Mukai & Iwata, 1991). In this way, glucocorticoids act by removing superfluous or autoimmune-prone components. Thus, the increase in glucocorticoid's concentrations to immunosuppressive levels, following the first wave of activation have the teleological purpose of sculpting and restraining the immune response, preventing its overshooting (Sapolsky, Romero & Munck, 2000).

Figure 8: the permissive and suppressive actions of glucocorticoids

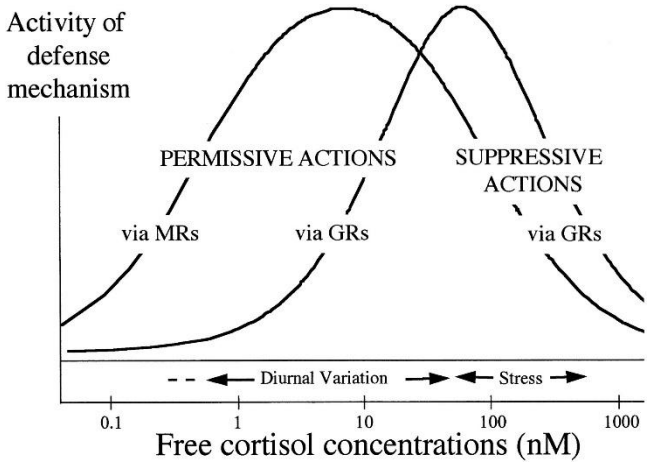

Figure 8: GRs-glucocorticoid receptors; MRs-mineralocorticoid receptors
Adapted from Sapolsky, Romero & Munck, 2000.

Neurobiologic effects: In the brain, glucocorticoids decrease glucose utilization by causing a translocation of glucose transporters from the cell´s membrane to the cytoplasm and by decreasing the levels of glucose transporter mRNA. These effects are evident at high stress doses of glucocorticoids and are mediated by the GCR. They can be categorized as immunosuppressive, because they serve to counteract and limit brain´s glucose uptake and utilization. Glucocorticoids also stimulate appetite, which serves to counteract the anorectic effects of early stress response and to prepare the body for future stressor events, by helping it to replenish its reserves. The initial concentrations of glucocorticoids (e.g. permissive action) are also permissive to catecholamine action in enhancing memory formation. This makes sense in evolutionary terms because once the stressor has occurred, it is evolutionary useful for the body to acquire and store knowledge about it. The stored information can then serve in the future to help the host in preventing or better adapting future stressors. In contrast the immunosuppressive effects of glucocorticoids seem to disturb memory formation. The significance of this finding is uncertain at the moment.

2.10.4. Other hormonal responses to HS

AVP, renin and angiotensin

The release of AVP by the posterior pituitary following HS has been known for decades (Hock, Su & Lefer, 1984). AVP release is stimulated by low blood pressure and low atrial

filling. AVP has several important effects in the pathophysiology of HS. In the kidney it promotes water reabsorption and the production of concentrated urine (Desborough, 2000). AVP also possesses vasoconstrictive effects, which help to divert blood from non-vital to vital organs (Schadt & Ludbrook, 1991). However, the impact of AVP is not as immediate as catecholamines because its peak levels are only reached after the first 2 hours of the stressor´s onset. In addition, its vasopressor effects have a maximal duration of 6 hours.
Hemorrhage also induces the release of renin and consequently the activation of the renin-angiotensin-aldosterone axis. This contributes to water reabsorption and volume expansion (Shackord, Norton, Ziegler & Wilner, 1988). In addition, angiotensin II also contributes to the hemodynamic compensatory changes to HS, by interacting with the adrenergic vasoconstrictor system (Korner, Oliver, Zhu, Gipps & Hanneman, 1990). The renin-angiotensin-aldosterone axis system is much more slower to react than other endocrine mechanisms and only reaches its maximum effects at 12-24 hours post-injury.

The Thyroid axis

Thyroxine (T4) and tri-iodothyronine (T3) are secreted into the circulation from the thyroid gland under the influence of the thyroid–stimulating hormone (TSH), which is released from the adenohypophysis (Desborough, 2000). Small amounts of inactive, reversed T3 are also produced from the thyroid. T3 is formed in the tissues by monodeiodination of T4. Thyroid hormones mainly circulate ligated to proteins. These are thyroid-binding globulin, albumin and thyroxine-binding pre-albumin. They are also present freely dissolved in plasma, although at very low concentrations. The free thyroid hormones in plasma are metabolically active. Thyroid hormones stimulate oxygen consumption by most metabolically active tissues in the body, except spleen, brain and anterior part of the pituitary. They also increase carbohydrate absorption by the gut, stimulate the central and peripheral nervous systems and at a long term, influence growth and development.
Critical illness is associated with reduced TSH and thyroid hormone secretion and with changes in peripheral thyroid hormone metabolism, resulting in low serum T3 and high reversed T3 and changes in tissue thyroid hormone levels (Peeters et al., 2005a; Peeters et al., 2005b). The presence of a 'low T3 syndrome' in the setting of nonthyroidal illness has long been recognized as the 'euthyroid sick syndrome' (Wyne, 2005). In euthyroid sick syndrome the first and most consistent thyroid hormone abnormality is a decrease in T3 levels. With increased severity and duration of illness, T4 levels are decreased as well, in part due to a decrease in T4 production. Thyrotropin levels in these patients are typically within or below the reference range during the acute phase of critical illness and only rise above normal levels during recovery (Ho, Chapital & Yu, 2004). The pathophysiology of

euthyroid sick syndrome it is not yet completely understood but maybe related with inhibitory effects of cytokines (van Haasteren et al., 1994) and catecholamines in the thyroid axis (Desborough, 2000). Because glucocorticoids can decrease TSH and T3 levels (Cavalieiri, Castle & McMahon, 1984), the increased levels of glucocorticoids characteristic of the stress response can be involved in its pathophysiology as well. The simultaneous presence of hypothyroidism and adrenal insufficiency in patients with HS has also been reported (Ho, Chapital & Yu, 2004).

If "euthyroid sick syndrome" should be treated remains a controversial topic (Wyne, 2005). Studies have shown that critically ill patients with low levels of THS, T4 and T3 and which fail to improve these to normal levels are more likely to die (Peeters et al., 2005b). In addition, experimentally it has been demonstrated that TRH supplementation has beneficial effects in experimental HS. In fact, the administration of TRH was associated to increases in arterial blood pressure, cardiac contractility and epinephrine levels (Zheng, Chen & Hu, 1992). The beneficial effects of TRH are believed to be dependent from its interaction with the adrenergic system, in particular due to its stimulation of the sympathomedullary system to secret epinephrine and by increasing sensitization to beta-adrenergic and dopamine receptors to cathecolamines (Zheng, Chen & Hu, 1992). In addition, TRH counteracts the effects of the endogenous opioid system, possibly by decreasing the expression of brain opioid receptors (Liu Hu, Chen, Lu & Yan, 1997).

Atrial natriuretic factor (ANF)

ANF is a peptide produced by mammalian atrial cardiomyocytes and which plays a major role in fluid and electrolyte homeostasis, control of vascular tone, and regulation of blood volume. It achieves these effects through its potent natriuretic, diuretic, and vasorelaxant properties in euvolemic and hypervolemic states (Needleman & Greenwald, 1986). Stimuli for the release of ANF include tachycardia, atrial distention and hyperosmolarity (Eskay, Zukowska-Grojec, Haass, Dave & Zamir, 1986). It has been shown that HS leads to an increase of ANF in swine (Shackord et al., 1988). By contray, in rats ANF seems to decrease (Kudo, Kudo, Oyama & Matsuki, 1989; Chiu et al., 1987).

The increased ANF levels following HS can be due to several factors. One is its decreased degradation due to plasma volume contraction (Shackord et al., 1988). Another is atrial distension following fluid resuscitation (Kudo et al., 1989). Finally it can result from increased levels of other hormones such as catecholamines and vasopressin.

ANF is considered to be one of the natural antagonists to AVP (Shackord et al., 1988) and this effect may be important in HS. In fact, ANF may have a physiologic role in the response to hemorrhage, by preventing that endogenous vasoconstrictors such as AVP which are,

released to compensate blood loss, promote excessive vasoconstriction (Shackord et al., 1988). Glucocorticoids also prevent the decrease in ANF associated to HS in rats (Kudo et al., 1989). Thus the maintenance of ANF levels may be one of the mechanisms by which glucocorticoids prevent AVP from overshooting in HS.

The opioid system

Increased production of POMC-derived β-endorphin as part of the acute stress response was already mentioned. Increased levels of β-endorphin have been described in fixed-volume models of HS (Molina, 2002). β-endorphin is an opioid peptide of 31 amino acids which belongs to one of the three families of endogenous opiate peptides, the endorphin family. The 2 other families are the enkephalin and the dynorphin families. Enkephalins and dynorphins are peptides derived from preproenkephalin A and preproenkephalin B, respectively (Jochem, Josko & Gwosdz, 2001). Opioid peptides act through ligation to several classes of G-protein coupled opioid receptors named as μ, κ and δ and the recently described nociceptin/orphanin FQ peptide receptor (NOP-R) (Witkin et al., 2014). In the case of the first three types of receptors, ligand binding induces the inhibition of adenylyl cyclase, decreasing intracellular AMPc levels and protein kinase A activity (Molina, 2005). Enkephalins have been considered the endogenous ligands for δ opioid receptors, β-endorphin and met-enkephalin for the μ receptors and dynorphins for the κ receptors (Standifer & Pasternak, 1997). Endogenous opioid peptides and opioid receptors have been found in many areas of the CNS, including the hypothalamic nuclei (preoptic nucleus, paraventricular nucleus and lateral hypothalamic nucleus), hippocampus, the parabrachial, dorsal tegmental, vestibular and raphe nuclei of pons, *medulla oblongata* and spinal cord dorsal grey (Jochem, Josko & Gwosdz, 2001). They have also been fund in several areas outside CNS, including immune cells.

Experimental and clinical studies have shown that the endogenous opioid system is activated in response to injury in parallel with HPA and sympathetic nervous system axis activation (Molina, 2005). This has been demonstrated to occur also in HS (Molina, 2002). In teleological terms, it is assumed that the function of increased opioid activation after injury is to provide anti-nociception (Jungkunz, Engel, King & Kuss, 1983) and the bio-behavioural feeling of well-being (Henry, 1982). In fact, it is believed that the endogenous opioid system, especially through the ligation to μ-opioid receptors and to a less extent, to δ-opioid receptors, is the main responsible for the development of stress-induced analgesia in rats (LaBuda, Sora, Uhl & Fuchs, 2000; Wiedenmayer & Barr, 2000) and Humans (Willer, Dehen & Cambier, 1981). Interestingly, in rats HS-induced analgesia seems to depend more from

an integral activity of the HPA axis than from the effects of endogenous opioids (Fukuda, Nishimoto, Miyabe & Toyooka, 2007; Fukuda, Nishimoto & Toyooka, 2005).

In addition it is now known that the endogenous opioid system also modulates the stress response to injury by regulating the normal function of the cardiovascular, immune and endocrine systems. In the cardiovascular system, the activation of the endogenous opioid system seems to counteract the effects of increased SNS activity. In effect, endogenous opioids are known to decrease the release of catecholamines, especially noradrenaline, by ligation to pre-synaptic μ, κ, and δ receptors. They also interact with adrenergic receptor function in target organs such as the heart, interfering with its function and by increasing vagal activity (Molina, 2005). Experimental studies have shown that β -endorphin is associated to decreased blood pressure in animals subjected to hemodynamic challenge (De Jong, Sandor, Cox-van Put, van den Berg & van Giersbergen, 1989). In addition, since the decade of 80 of the last century it is known that the administration of naloxone, a general opioid antagonist, has beneficial effects in blood pressure and hemodynamic status of animals submitted to HS (van den Berg, van Giersbergen, Cox-van Put & De Jong, 1991). Interestingly, there is evidence that naloxone´s beneficial actions in HS are partially due to its association with endogenous glucocorticoid activity (Eijgelshoven, De Kloet, Van den berg & Van Giersbergen, 1991).

Endogenous opioids also possess several immunological effects. It is believed that opioid´s immune effects result from its interaction with the HPA and SNS axis or from its direct activity upon immune cells (Nugent, Houghtling & Bayer, 2011; Molina, 2005). The latter, depends from several factors including the dosage and timing of administration of the opioid (mainly studied in exogenous opioid peptides), the type of receptor which the opioid preferentially ligates and the cell type which possesses that receptor (Schwacha, 2008). Depending from these factors both endogenous and exogenous opioids can promote shifts in circulating lymphocyte phenotype and can suppress many components of the immune response, including NK cell activity, neutrophil complement and immunoglobulin receptor expression, chemokine-induced chemotaxis and phagocytosis (Molina, 2005). Although the immune effects of opioids are now clearly established by *in vivo* and *in vitro* experiments, the physiological role of these immune effects and its clinical consequences are still being debated (Al-Hashimi, Scott, Thompson & Lambert, 2013; Schwacha 2008). In the response to injury, some authors suggest that the immune actions of endogenous opioids regulate SNS´s immune effects in the local immune response to injury (Molina, 2006).

The endogenous opioid system is also known to modulate several endocrine functions, including HPA axis´s activity. Opioids increase HPA activity, especially through the stimulation of μ-receptors (degli Uberti et al., 1995; Iyengar et al.¸1987). Of particular interest is the recognition that the endogenous opioid system seems to be involved in the catabolic

state that follows injury, by promoting an insulin-resistant state and subsequent hyperglycemia (Molina, 2006).

2.11. Relative adrenal insufficiency (RAI), also known as critical illness-related corticosteroid insufficiency (CIRCI)

Now that the normal stress response has been described we are in condition to approach the complex changes that occur in the HPA axis during critical illness. One of HPA´s axis dysfunctions which have recently been described includes RAI which is also known by the term CIRCI.

Any type of critical illness or trauma results in the loss of glucocorticoids diurnal variation and an increase in its levels. This increase is induced by CRH and ACTH coupled to an inhibition of the negative-feedback associated to cortisol (Mesotten, Vanhorebeek & Van den Berghe, 2008). The increased levels of certain cytokines (see below) also contribute to augment glucocorticoids levels, by inducing an activation of the HPA axis. Cytokines also affect glucocorticoids activity by modulating the activity of enzymes such as 11β-HDS and the number and/or affinity of GCRs (Marik & Zaloga, 2002). In addition, due to a decreased hepatic production and increased elastase activity, the levels of corticosteroid-binding globulin (CBG) are decreased, leading to increased levels of free glucocorticoid and consequently an increase in its biological activity (Beishuizen, Thijs & Vermes, 2001). In chronic critical illness, glucocorticoids also tend to be elevated, but contrary to the acute situation, ACTH levels are normal or low. This suggests that in chronic critical illness, increased ACTH levels do not play a role in the increased levels of cortisol.

Increased mortality due to critical illness has been associated to both very high and very low levels of glucocorticoids (Rothwell & Lawler, 1995), which suggests that a normal and balanced HPA axis is essential to achieve survival after critical illness. The reason why increased levels of glucocorticoids are associated to worse prognosis in critically ill patients is not yet completely understood although several hypotheses have been advanced. One possibility is that the high levels of glucocorticoids reflect the severity of the stressor. This hypothesis makes sense because it is reasonable to assume that a severer stressor impairs more significantly hemostasis and consequently leads to a worse outcome. Another possibility is that increased levels of glucocorticoids increase the susceptibility to infection.

On the other hand, a pathologically lower level of glucocorticoids also represents a dysfunction of the HPA axis associated to increased mortality. In this case, the increased mortality most probably results from an inadequate stress response. A pathological low level of glucocorticoids during critical illness is one of the aspects of RAI or as it is more recently known, CIRCI. Although CIRCI has been increasingly recognized in the last three decades,,

several aspects of its diagnosis, management and pathophysiology are still unknown. These and other subjects will be discussed in the subsequent subsections.

2.11.1. Definition

CIRCI is currently defined as an inadequate intracellular glucocorticoid anti-inflammatory activity for the severity of the patient's illness. CIRCI can result from either insufficient availability of glucocorticoids to the cell or from intracellular resistance/insensitivity to glucocorticoids (despite elevated levels of circulating cortisol) (Marik et al., 2008).

2.11.2. Pathological consequences

CIRCI has been associated to increased patient´s morbidity and mortality in sepsis, septic shock and trauma patients (Hohl et al., 2010; Marik et al., 2008). A study in trauma patients revealed that those diagnosed with acute adrenal insufficiency and which were not treated with replacement steroids had higher mortality, longer hospital length of stay, intensive care unit (ICU) days, and lower ventilator-free days (Guillamondegui et al., 2009). The presence of CIRCI has also been associated to increased probability of death in HS (Stein et al., 2013). On the other way round, there are also studies which reported a lack of influence of CIRCI in patient´s outcome. This has been reported for example, in a study where the incidence of CIRCI and its effects in outcome were assessed in patients subjected to rapid-sequence-intubation (RSI) (Freund et al., 2014).

2.11.3. Prevalence of CIRCI

CIRCI has been identified in several critically illnesses including sepsis and septic shock (Burry et al., 2013; Annane et al., 2000;), HS (Cotton et al., 2008; Beeman, Veverka, Lambert & Boysen, 2005¸ Hoen et al., 2002), liver cirrhosis (Tsai et al., 2006), pneumonia and ARDS (Annane, Meduri & Marik, 2008), pancreatitis (Peng et al., 2009), cardiac arrest (Kim et al., 2011), acute aneurysmal subarachnoid hemorrhage (Weant, Sasaki-Adams, Dziedzic & Ewend, 2008), spinal cord injury (Weant, Sasaki-Adams, Kilpatrick & Hadar, 2008), and head trauma (Olivecrona, Dahlqvist & Koskinen, 2013). CIRCI has also been described in experimental models of hemorrhage in rats with portal hypertension (Lee et al., 2014), sepsis and septic shock (Hu et al., 2009), HS (Rushing et al., 2006; Wang et al., 1999), traumatic brain injury (Chen et al., 2013) and ANP (Yu et al., 2012).
The prevalence of CIRCI is high in critically ill patients, varying between 54-70% of patients, depending from the nature of the underlying disease (Yang et al., 2014; Burry, Little, Hallett,

Mehta, 2013; Kromah et al., 2011). CIRCI´s incidence in sepsis and septic shock has been reported to vary between 20-60% (Marik, 2009; Yang et al., 2007; Annane et al., 2006). In post-cardiopulmonary arrest patients CIRCI was described in 52% of patients (Pene et al., 2005). In trauma, the incidence of CIRCI was initially believed to be uncommon based in an initial study which reported a low incidence of the condition in severely injured patients (Beeman et al., 2005). However more recent studies questioned these findings, by demonstrating that CIRCI can affect up to 60% of patients with trauma (Yang et al., 2014; Guillamondegui et al., 2009; Cotton et al., 2008). Interestingly in each clinical condition which has been studied, there are subpopulations which seem more prone to develop CIRCI than others. For example, in septic shock, the presence of bacteremia, female gender and concomitant ARDS seem to be more associated to CIRCI´s development (Salgado, Rocco & Rosso Verdeal, 2008). In addition, the incidence of absolute and relative adrenal insufficiency in children with septic shock reaches values as high as 80% (Hebbar, Stockwell, Leong & Fortenberry, 2011).

CIRCI in HS

CIRCI seems also to be common in HS, with studies reporting an incidence from 47 to 80% (Stein et al., 2013; Rushing et al., 2006; Hoen et al., 2002). Interestingly, Stein et al. (2013) were able to demonstrate that in HS CIRCI may occur in a hyperacute presentation, associated to a high mortality (Stein et al., 2013). The occurrence of CIRCI associated to HS has been also described in rat experimental models (Rushing et al., 2006; Wang et al., 1999).

2.11.4. Etiology of CIRCI

Several etiologies can be associated to the inability of the adrenal gland to produce cortisol in response to stress (Marik & Zaloga, 2002). These include hypothalamic and pituitary disorders and destructive diseases of the adrenal gland. Examples of the former in Humans, include pituitary or metastatic tumors, pituitary surgery or radiation, empty-sella syndrome, craniopharyngioma, sarcoidosis, histiocytosis, post-partum pituitary necrosis, HIV infection and head trauma. Examples of diseases causing acute adrenal gland destruction include auto-immune adrenalitis, HIV and cytomegalovirus infection, antiphosphoslipid syndrome, systemic fungal infections, tuberculosis, metastatic carcinoma (breast, lung, and kidney) and acute adrenal hemorrhage secondary to disseminated intravascular coagulation, meningococcemia and anti-coagulation drugs. Interestingly, the occurrence of adrenal injury following abdominal blunt trauma has not been shown to be a predictor of CIRCI in traumatic

patients (Castaldo et al., 2008). Several drugs are also known to interfere with adrenal function including chronic glucocorticoid therapy, ketoconazole, etomidate, megestrol acetate, metyrapone and mitotane. Drugs such as rifampin and phenytoin are also associated to adrenal insufficiency because they increase cortisol metabolism. Hypothermia is also known to decrease adrenal hormone production.

However this list of causes represents just a small minority of CIRCI´s cases that occur in critically ill patients. The first comprehensive study that documented the presence of CIRCI in critically ill patients was the study of Briegel and colleagues, which reported that in 20 patients with septic shock, 13 had a stress level of cortisol lower than 25 µg/dL. This value was considered low taking in account the severity of the condition (Briegel et al., 1996). Since then there is increasing evidence that CIRCIs occurs in critically ill patients and currently it is believed that in most of cases it has a multifactorial origin. Initially it was believed that CIRCI resulted solely from decreased cortisol production. Proposed mechanisms for the decreased production include decreased levels of CRF and ACTH and the presence of suppressive factors, namely cytokines such as TNF-α, IL1 and IL6. In fact, when cytokines are present in large amounts for extended periods of time, they can induce a state of adrenal exhaustion (Marik & Zaloga, 2002; Zaloga, 2001). In addition, decreased cortisol production was also found to result from substrate deficiency. In the adrenal gland, high-density lipoprotein is the preferred cholesterol source to produce hormones such as cortisol. It has been shown that high-density lipoprotein can be substantially reduced in patients with critical illness (Marik, 2009). A decreased adrenal gland sensitivity to both endogenous and exogenous ACTH in critical illness has also been found (de Jong et al., 2015). These lead to corticosteroid tissue resistance and inadequate levels of circulating free cortisol (Marik, 2009).

In recent years our understanding about the behavior of the HPA axis´s in critical illness has refined our understanding of CIRCI. Studies have shown that some critically ill patients can have higher serum cortisol levels and that these are not the result of increased production but rather from a decrease in its metabolism (Boonen et al., 2013). The latter seems to result from a decrease in the activity and expression of kidney and hepatic metabolizing enzymes and can represent an adaption to critical illness (Boonen et al., 2013). Increased cytokine and serum bile acids levels can also contribute to these changes (Peeters, Boonen, Langouche & Van den Berghe, 2015).

It has also been suggested that increased cortisol levels can lead to a decreased ACTH production and secretion and to deleterious consequences in the function and structure of the adrenal gland. Both factors ultimately can lead to decreased cortisol production (Peeters et al., 2015).

The importance of the ultradian rhythm in cortisol and ACTH secretion for the function of glucocorticoids is a well-known phenomenon (Gibbison, Angelini & Lightman, 2013). Because the HPA axis functions accordingly to ultradian rhythms, during the day glucocorticoids are released in a pulsatile manner. It is now recognized that this ultradian pulsatility is important for maintaining a proper homeostatic regulation and responsiveness to stress. Critical illness leads to a decrease in nocturnal and pulsatile secretion of glucocorticoids (Veldhuis, Keenan & Pincus, 2008). It is possible that this phenomenon also contributes to the abnormal function of the HPA in critical illness.

More recently, it has been recognized that glucocorticoid resistance which occurs at tissue level can be a cause of CIRCI in sepsis and ALI as well, in a similar way to what occurs with insulin in *diabetes mellitus* type II. Tissue glucocorticoid resistance is a phenomenon previously recognized in chronic obstructive pulmonary disease, severe asthma, systematic lupus erythematosus, ulcerative colitis and rheumatoid arthritis (Marik, 2009). The mechanisms behind tissue glucocorticoid resistance are still being elucidated but they include: downregulation in the number of GCRs, especially at the cell´s nucleus; decreased binding affinity of GCR to glucocorticoids and other post-receptor alterations (Meduri, Muthiah, Carratu, Eltorky & Chrousos, 2005; Molijn et al., 1995); and increased cytokines levels such as TNF-α, IL1 and Macrophage-Inhibitory Factor (Arafah, 2006).

2.11. 5. Clinical signs of CIRCI

The clinical consequences of CIRCI result from an exaggerated proinflammatory immune response. One of its cardinal manifestations is hypotension refractory to fluid and vasopressor administration (Marik, 2009). When this occurs it should raise the suspicion of underlying CIRCI (Boonen & Van der Berghe, 2016).

CIRCI should be considered in every ICU patient who requires vasopressor support. Patients with CIRCI have a hyperdynamic circulation and its SVR, CO and pulmonary capillary wedge pressure can be low, normal or high, reflecting the combination of CIRCI with the underlying disease. CIRCI should also be suspected in patients with progressive ALI.

Laboratory abnormalities that raise the suspicion of the condition include eosinophilia and hypoglycemia. However, contrary to what occurs with chronic adrenal insufficiency, the development of hyponatremia and hyperkalemia is uncommon (Marik, 2009).

2.11.6. Etomidate and CIRCI

Etomidate is an anesthetic agent introduced in clinical practice in 1972 and since then, it has been recognized as being able to maintain cardiovascular stability in hemodynamically

unstable critically ill patients (Bergen & Smith, 1997). Beneficial effects associated to etomidate include: lack of inhibitory effects in the sympathetic tone or myocardial function; minimal influence in blood pressure or heart function at typical anesthetic induction doses; decreased incidence of apnea when compared with propofol or barbiturates; absence of interference in the sympathetic responses to laryngoscopy and intubation; absence of histamine release and low incidence of allergic reactions (Forman, 2011). All this properties quickly made etomidate one of the most frequently used drugs in critically ill patients. Initially etomidate was marketed for use in both bolus and constant-rate infusion. However studies performed in the 80s of the XX Century clearly demonstrated that its use by constant-rate infusion or in multiple doses was associated to an increased risk of patient´s morbidity and mortality (Watt & Ledingham,1984). The latter were attributed to its adrenal suppressive properties, which result from the inhibition of the cytochrome P450 enzyme 11β-hydroxylase (Forman, 2011). Nevertheless because adrenal suppressive properties were believed to be minimal after single bolus administration, etomidate remained to be used in this form of administration. It is currently considered as the hypnotic drug of choice for RSI and intubation by many emergency physicians (Forman, 2011).

In the first decade of the XXI century, after the work in septic shock by Briegel et al., (1999) and Annane et al., (2000) the concept of CIRCI in critically ill patients entered the clinical arena (Annane et al., 2000; Briegel et al., 1999). In 2002, the group of Annane, while investigating the effects of steroid supplementation in patients with septic shock, reported that patients who had received a single dose of etomidate demonstrated a lack of cortisol response to ACTH stimulation (Annane et al., 2002). Accordingly to the authors, the use of etomidate in critically ill patients was considered a confounding factor for the diagnosis of CIRCI (or RAI, as it was called at the time). This launched the debate in the scientific community about the safety of using etomidate as a bolus in critically ill patients.

It is now clear that etomidate is associated to CIRCI in critically ill patients, even after the administration of a single dose and especially in conditions of substrate deficiency (Molenaar et al., 2012). Etomidate´s associated CIRCIs have been found in several pathological conditions. For example, CIRCI was found in 76% of patients with sepsis which received a single bolus of etomidate (Mohammad et al., 2006) and similar results were obtained in trauma (Freund et al., 2014; Cotton et al., 2008). Etomidate was also associated to CIRCI in burned patients (Mosier, Lasinski & Gamelli, 2015). In addition to be common, it was recognized that the duration of etomidate-associated adrenal suppression is more prolonged in critically ill patients. In fact, studies have shown that etomidate administered as single bolus is associated to suppression of the adrenal production of glucocorticoids which may last until 48 hours in critically ill patients (Vinclair et al., 2008; Lundy, Slane & Frizzi, 2007).

The clinical significance of etomidate´s "pharmacologic adrenalectomy" and etomidate-associated CIRCI for patient´s outcome is still unclear and subject to a lot of controversy. This is due in part from the lack of clear scientific evidence (van den Heuvel et al., 2013). Several studies are available that have addressed this question with mixed results. The next section will focus on the evidence that links etomidate to increased mortality. The following section will display the evidence for the contrary.

Etomidate increases mortality

In a-priori sub-study of the CORTICUS multi-center, randomized, double-blind, placebo-controlled trial of hydrocortisone in septic shock (Cuthbertson et al., 2009), the use of a single dose of etomidate in the first 72 hours before study inclusion was associated to several side-effects, including increased patient´s mortality. Also, Hildreth et al., (2008), found that trauma patients to whom etomidate was administered had longer hospital and ICU lengths-of-stay and increased days spent in the ventilator. In trauma patients, Asehnoune et al., (2012) were able to identify an increased risk of the incidence of pneumonia and ARDS in patients treated with etomidate (Asehnoune et al., 2012). Etomidate administration was also associated to increased mortality in critically ill patients (Sunshine et al., 2013), and in adult (Cherfan et al., 2011) and pediatric patients with sepsis (den Brinker et al., 2008). Several meta-analysis also reached the same conclusions. For example Albert, Ariyan & Rather, (2011) demonstrated that the use of etomidate was associated to an increased risk ratio for developing adrenal insufficiency of 1.64 (range 1.52-1.77; 14 studies, 2,854 patients, $p<0.0001$, $I(2)=88\%$) and an increased risk ratio of mortality of 1.19 (1.10-1.30; 14 studies, 3,516 patients, $P<0.0001$, $I(2)=64\%$) (Albert, Ariyan & Rather, 2011). When a reanalysis was performed to take in account the nature of the underlying condition, increased mortality due to etomidate was especially observed in septic patients. Another meta-analysis, this time only addressing septic patients also found an increased incidence of CIRCI and mortality in patients that received etomidate (Chan, Mitchell & Shorr, 2012). Finally, in the most recently available meta-analysis, Bruder et al., (2015) concluded that etomidate administration in critically ill patients is associated to increased risk of developing CIRCI and MOF (Bruder et al., 2015).

How etomidate increases mortality is still undefined although this is believed to be related with the development of CIRCI. In turn, how CIRCI may augment mortality is still being debated. One possibility is that the occurrence of etomidate-associated CIRCI leads to pathological levels of cytokines and a prolonged state of SIRS (Kwon et al., 2010). This in turn would lead to increased rate of organ dysfunction and infectious complications. In fact, it was demonstrated in trauma patients the prolonged SIRS predicts the development

ofnosocomial infection (Bochicchio et al., 2002). The decreased glucocorticoid levels can also directly increase the risk of infection, because normal levels of these hormones are necessary to mount a normal response to infectious agents (Sapolsky, Romero & Munck, 2000). Corraborating this fact, as as already mentioned, etomidate was associated to increased rate of pneumonia in trauma patients (Asehoune et al., 2012).

Interestingly etomidate has also direct effects in inflammation. Etomidate has been shown to increase IL6 in women submitted to hysterectomies (Jameson et al., 1997) and to induce pro-inflammatory cytokine production in whole blood cultures stimulated with LPS (Larsen et al., 1998). It can also interfere with IL10 and with circulating lymphocytes levels (Cherfan et al., 2011) and mitigate the release of pro-inflammatory cytokines by rat macrophages exposed to LPS (Liu et al., 2015). How etomidate exerts these effects is incompletely understood although it is known that the drug can inhibit NFκB translocation (Zhang et al., 2015). Interestingly this inhibition of NFκB translocation was associated to an increased mortality in a rat model of sepsis. It is possible that the direct impact of etomidate in inflammation plays a role in its contribution to increased mortality. However this hasn´t yet been proven.

It may also be hypothesized that the etomidate-associated lack of glucocorticoids can increase mortality by increasing hemodynamic instability, due to the natural role of glucocorticoids in normal cardiovascular responses. However the use of etomidate was not associated to increased vasopressor requirements in several studies (Alday et al., 2014; Elliot, Brown & Kuo, 2012).

Another factor which can explain the role of etomidate in increased mortality is the decreased levels of other hormones, in particular of aldosterone due to etomidate´s adrenal suppressive effects. In fact, in cirrhotic patients etomidate administration increased mortality and this was attributed to etomidate-associated hypoaldosteronism (Ulleras, Ohlsson & Oskarsson, 2008).

Etomidate may also affect ACTH secretion, with some studies showing that it increases ACTH release (Preziosi and Vacca, 1982) and others the opposite (Stalla et al., 1989). If these changes in ACTH secretion caused by etomidate have any clinical significance remains undetermined.

Etomidate does not increases mortality

There are also several retrospective studies which suggested that etomidate-associated CIRCI was not associated to increased incidence of side-effects, including mortality. Mohammad et al. (2006), in a retrospective study of 152 septic shock patients which received etomidate demonstrated that these had a higher rate of CIRCI but not of increased mortality when compared with controls (63 vs. 55%, p = 0.45) (Mohammad et al., 2006). Also

Ray & McKeown (2007), in a review of 159 septic shock patients did not found any difference in mortality between patients that used etomidate with those anesthetized with other agents (Ray & McKeown, 2007). Tekwani, Sweis, Rzechula & Kulstad, (2010) also did not found any difference in hospital length of stay in septic patients to whom etomidate was used for RSI (Tekwani, Sweis, Rzechula & Kulstad, 2010). More recently, in a retrospective analysis of septic patients, McPhee et al., (2013) did not report increased mortality in septic patients which received etomidate (McPhee et al., 2013). The absence of significant adverse side effects associated to etomidate was also demonstrated in studies which evaluated other populations of critically ill patients. These include patients which required RSI in the Emergency Department (Baird et al., 2009) and patients admitted to ICU after emergency laparotomy (Ray, Hay & McKeown, 2010).

Several prospective studies have also demonstrated a lack of increased mortality associated to etomidate. Jabre et al. (2009), in a study which involved 655 trauma and septic patients showed no increased mortality rate with the use of etomidate when compared with ketamine (Jabre et al., 2009). Again, etomidate was associated with a significant higher rate of adrenal insufficiency (odds ratio 6.7; 95% CI: 3.5-12.7). However the 28-day mortality for etomidate was 35% versus 31% for ketamine ($p = 0.36$). A subgroup analysis of the 76 septic patients showed no statistically significant difference in mortality with etomidate vs. ketamine with an odds ratio of 1.4 [95%CI 0.5-0.35] (Jabre et al., 2009). Tekwani, Watts, Rzechula, Sweis & Kulstad, (2009) also did not report increased mortality in septic patients induced with etomidate (Tekwani, Watts, Rzechula, Sweis & Kulstad, 2009).

Finally a meta-analysis performed by Hohl et al., 2010 also did not demonstrated increased mortality associated to etomidate administration, although the authors warned that the studies which composed their meta-analysis were not powered enough to detect a difference in hospital, ventilator, or ICU length of stay or mortality (Hohl et al., 2010) . To add more fuel to the debate, a more recent meta-analysis showed that reducingetomidate use for intubation in trauma patients was not associated to differences in mortality, ICU days, or hospital length of stay(Banh et al., 2012). However it was evident that etomidate increased the rate of episodes of hypotension within 24h of presentation (Banh et al., 2012).

What can we conclude about etomidate´s safety in critically ill patients?

Most probably the different conclusions reached by the different studies result from methodological issues including the lack of enough statistical power. In addition, it is possible that etomidate has effects which are different accordingly to the study population. For example most studies which reported increased mortality involved septic patients. In

contrast, the evidence for increased mortality in trauma patients is more limited. Besides, not all studies have reported effects in hospital length of stay, mortality, ICU stay and other outcome measurements. Even in the most complete and recent meta-analysis to date and which was already mentioned (Bruder et al., 2015), the authors acknowledged the existence of several limitations. They found that most studies used to perform the meta-analysis were of moderate quality. The authors also declared the need to perform new and better studies in this matter to finally clarify the safety of etomidate in critically ill patients.

Etomidate in HS

Etomidate is considered an ideal agent of intubation in patients with HS due to his beneficial hemodynamic profile, a fact confirmed by several experimental studies. In rats submitted to HS by blood loss to reach a MAP of 30 mmHg, an etomidate bolus of 3 mg/kg I.V. did not to increased mortality when compared with controls (Peterson et al.¸1985). In addition, in a study with dogs submitted to hypovolemia, Pascoe et al., (1992) reported that etomidate administered as a bolus was associated to minimal cardiovascular changes (Pascoe et al.,1992). In another experimental study using dogs, etomidate was able to maintain hemodynamic stability in severe HS treated with NaCl 0.9% or NaCl 7.5% (Fraga et al., 2006). In contrast, in some experimental models etomidate was shown to increase tissue DO_2 (Van der Linden et al., 2000). The beneficial effects of etomidate for RSI in HS were also demonstrated in several clinical studies (Zed, Abu-Laban & Harrison, 2006; Bergen & Smith, 1997).

Several experimental studies have demonstrated that HS has the potential to influence etomidate´s pharmacokinetics and pharmacodynamics. For instances it is known that both moderate and severe states of HS decrease the clearance and distribution volume of etomidate (Johnson et al. 2003; de Paepe et al., 1999). HS has also the potential to induce pharmacodynamic changes although these are less likely to occur (de Paepe et al., 1999). These changes of etomidate´s pharmacokinetics and pharmacodynamics may increase its potency in states of hypovolemia and consequently a dose adjustment may be necessary in HS (Johnson et al., 2003).

To our knowledge few experimental studies have addressed the consequences of etomidate-adrenal suppressive properties in HS. In a study of hemorrhage in dogs, Fraser, Watt, Gray, Ledingham & Lever (1984) reported that the use of etomidate, when compared with thiopentone and pentobarbitone, was associated to significant decreases in progesterone, 17alpha-hydroxyprogesterone, CS, cortisol, and aldosterone despite the massive increases in plasma ACTH concentration, renin, and angiotensin II (Fraser, Watt, Gray, Ledingham & Lever, 1984). However the authors did not reported any difference in outcome or incidence of side-effects associated to these changes. In another study where dogs were subjected to

hypoxia under enflurane anesthesia and to tialmilal or etomidate administration, the decrease in cortisol associated to etomidate did not affect the responses to hypoxia (Hirschman, 1991). In contrast, the cortisol deficiency induced by metomidate, a drug closely related to etomidate, facilitated the development of circulatory failure in the course of *Pseudomonas* spp bacteremia in anesthetized pigs (Neumann et al., 1989).

2.11.7. Laboratory diagnosis of CIRCI

The best laboratory method to diagnose CIRCI has been considerably debated. This debate is confounded by the fact that the levels of endogenous glucocorticoids which are considered adequate for the different types of critical illness are still unknown. In addition, astandardized definition about what constitutes a normal biochemical response to critical illness is still lacking. This may vary with the nature and duration of the critical illness (Arafah, 2006).
Several criteria have been proposed to diagnose CIRCI including the measurement of random cortisol levels and the results of stimulation tests to assess the functionality of whole or part of the HPA axis. However no consensus has been reached yet. Tests that evaluate the cortisol response include the ACTH stimulation test, the metyrapone test and the insulin tolerance test (by inducing hypoglycemia). The insulin tolerance test assesses the integrity of the HPA axis as a whole, and consists in the administration of insulin to decrease blood glucose concentrations, which then leads to increased HPA activity. The metyrapone test consists in the administration of metyrapone to decrease cortisol concentrations, which is followed by an assessment of the compensatory response of the HPA axis, which should include an increase in ACTH levels. These two tests are rarely assessed in critically ill patients because they can expose patients to severe side-effects. Traditionally the ACTH stimulation test and the measurement of total cortisol levels have been used to diagnose CIRCI in critically ill patients (Arafah, 2006). These will be described briefly in the following sections.

Measurement of total cortisol levels

The measurement of random cortisol levels has been advocated based in the knowledge that cortisol levels rise with the onset of a stressful stimulus (such as hypotension and pain). Thus the test in based in the assumption that the clinical changes induced by critical illness function as a stimulus for the adequate adrenal response to stress (Moraes et al., 2011).
In normal patients without critical illness it was found that a level of cortisol higher than 18 µg/dl has a high negative likelihood-ratio of adrenal failure. Unfortunately this cutoff level does not reflect adequately the endocrine milieu characterized by critical illness. In fact much

higher levels of cortisol are expected to occur in critical illness and consequently, in this context, any level of glucocorticoid higher than 18 µg/dl does not excludes the diagnosis of CIRCI. Most experts now agree that the finding of low baseline cortisol levels in critically ill patients has a high positive like-likehood ratio of CIRCI. Based on a seminal study performed by Annane and colleagues (2000), a random level of cortisol lower than 25 µg/dl was initially considered indicative of CIRCI. Using this value as cutoff, the sensitivity and specificity to diagnose CIRCI were found to be 0.55 and 0.56, respectively (Annane et al., 2000). In addition, most experts agree that during critical illness, the finding of cortisol levels lower than 10 µg/dL is considered low and levels higher than 33-34 µg/dL are considered high (Moraes et al., 2011). The cutoff value of 10 µg/dL as indicative of CIRCI has been found to have a high specificity in CIRCI diagnosis (specificity, 1; 95% CI, 1-1) (Annane et al., 2006) and it has been proposed as a cutoff for this purpose (Marik et al., 2008).

There are many pitfalls for the use of random total cortisol levels to diagnose CIRCI. Most of cortisol (90%) circulates bounded to plasma proteins, in particular CBG and albumin (Moraes et al., 2011). The levels of CBG and albumin decrease in critical illness which causes an artificial decrease in total cortisol levels (Beishuizen, Thijs &Vermes, 2001). For example, a study performed in septic patients demonstrated that patients with low albumin levels (lower than 2.5 g/dL) had lower variation in their levels of total cortisol after the ACTH stimulation test (Hamrahian, Oseni & Arafah, 2004).

Measurement of free cortisol levels

The pitfalls associated to total cortisol levels led some authors to suggest the measurement of free cortisol levels as a better indication of adrenal status (Hamrahian, Oseni & Arafah, 2004). Free cortisol can be measured in plasma after its separation from bound cortisol, calculated with an equation based on equilibrium binding or evaluated by cortisol/CBG ratio. Some authors suggested that the use of an equation to calculate free cortisol based only in CBG and total cortisol is less accurate because it does not take in account albumin levels (Moraes et al., 2011).Nevertheless not all experts agree with this suggestion. Their line of reasoning is that the use of albumin levels is not justified as this protein does not play a significant role in glucocorticoid transport (Molenaar et al., 2011).

Free cortisol can also be measured in salivary or urinary samples, although these methods cannot be easily applied in ICUs (Dolomie-Fagour & Corcuff, 2008). More recently and based on experimental models, a formula that estimates plasma free cortisol concentration based in the presence of elastase-cleaved and intact CBG has been proposed although the clinical experience with it is still limited (Nguyen et al., 2014).

Suggested cutoff values of free cortisol to diagnose CIRCI are a random value of free cortisol lower than 2.0 μg/dL or an increase in free cortisol after ACTH administration of less than 3.1μg/dL (Hamrahian, Oseni & Arafah, 2004). Several studies have been conducted to assess the value of free cortisol to diagnose CIRCI in critically ill patients. These were mainly performed in sepsis and at least in some the diagnosis of CIRCI seemed to be more accurate by using free cortisol than by using total cortisol levels (Ho et al., 2006; Hamrahian, Oseni & Arafah, 2004). However there are also studies that state the opposite (Bendel et al., 2008; Annane et al., 2006). In addition there are still experts that maintain the recommendation to use total cortisol to diagnosis CIRCI in sepsis, because this is highly correlated with free cortisol (Molenaar et al., 2011).

Although the measurement of free cortisol seems a logical approach, most hospitals do not have the technical methods to measure CBG and free cortisol. This fact and the lack of studies which confirm unequivocally its diagnostic value limit the widespread use of this method to diagnosis CIRCI.

The measurement of free cortisol levels for diagnosing CIRCI in septic patients was not recommend in the last Surviving Sepsis Campaign (Dillinger et al., 2012). Instead the latter suggested as a criteria of CIRCI diagnosis the finding of a random total cortisol level lower than 18 μg/dL. In addition the recommendations are that if levels of this magnitude are found in a patient with septic shock thansteroid therapy is indicated.

The high-dose ACTH stimulation test

The ACTH stimulation test consists in the administration of ACTH to stimulate the release of cortisol from the adrenal gland. The latter is measured at baseline and then at 30 and 60 minutes after ACTH administration. It is validated for the study of adrenal function in non-critical situations (Dickstein & Saiegh, 2008).

There are two types of ACTH test: the high and the low dose tests, where 250 μg and 1 μg of ACTH, respectively, are administered to the patient. The high-dose test was the first to be used to diagnose CIRCI in critically ill patients in the last decade of the XX century (Duggan, Browne & Flynn,1998; Bouachour et al., 1995). Annane et al. (2000) based on the results of their study, first proposed the cutoff values of variation of cortisol (the so called Δcortisol) of < 9 μg/dl to diagnose CIRCI in septic patients (Annane et al., 2000). In later study the same group also demonstrated that patients which had a Δcortisol < 9 μg/dl and which were treated with hydrocortisone had a lower mortality and a less time to shock reversal and vasopressor withdrawal than untreated patients (Annane et al., 2002).

In 2008, consensus guidelines established that the high-dose ACTH test should be one of the diagnostic standards to diagnose CIRCI (Marik et al., 2008). Since then many ICU´s around the World start to use the high-dose ACTH test to assess the need for hydrocortisone therapy in critically ill patients. However there are pitfalls associated to this test. One is that the dose of 250 µg of ACTH is supra-physiological, being far higher than the levels of ACTH which are frequently found in critically ill patients. These suprapharmacologic doses can lead to a supraphysiologic stimulation of the adrenal which in turn can overcome any underlying adrenal resistance to ACTH. In other words, by using the supraphysiologic doses onemay obtain an adequate response and believe that there is no dysfunction of the HPA axis, when in fact, there is. This is particularly worrying in the presence of an inadequate adrenal reserve. In addition, the supraphysiologic dose stimulates exclusively the adrenal gland, bypassing the hypothalamus and the pituitary gland and thus missing the rare cases where CIRCI results from hypothalamic or pituitary lesions (Arafah, 2006). Besides, as it has been discussed before, in most trials which reported the use of the high-dose ACTH test, total cortisol levels were the ones which were measured. In these conditions a low or blunted response to ACTH can just represent a low level of CBG and/or albumin (Arafah, 2006).. Finally, it has been argued that the test has high specificity and low sensitivity (Moraes et al., 2011). Interestingly, a recent study proposed the use of endogenous cortisol-ACTH ratio as a indicator of adrenal sensitivity. This study found this ratio to be useful in the diagnosis of adrenal dysfunction in critically ill patients (De Jong, Molenaar, Beishuizen & Groeneveld, 2015).

The low-dose ACTH stimulation test

Theoretically the lowdose ACTH test has one significant advantage in comparison with the high-dose ACTH test: it uses a dose of ACTH (1µg) which is similar to the physiologic levels of ACTH found in critically ill patients with sepsis (Beishuizen et al., 1999). However only a few studies have evaluated if this theoretical advantage is reproducible in clinical practice. In one study in septic shock, the abnormal response to low-dose ACTH test was defined as a Δcortisol lower than 9 µg/dl (Marik & Zaloga, 2003). Some patients were found to be unresponsive to the low-dose test, but responsive to the high-dose test. Interestingly, these were those which had a worse prognosis (Marik & Zaloga, 2003). The authors of this study suggested that the low-dose ACTH test was better in revealing the presence of adrenal dysfunction than the high dose ACTH test. In a retrospective study in septic patients, the use of the low dose ACTH test permitted to identify CIRCI in 70,8% of the study population (Burry et al., 2013). Finally a recent prospective study found a similar accuracy between the low-

dose and the high-dose ACTH tests to identify non-survivors and to predict time to vasopressor withdrawal in septic shock (Moraes, Friedman, Tonietto, Saltz & Czepielewski, 2012).

Although the use of the low-dose ACTH test to identify CIRCI seems promising, further studies are needed before this substitutes the high-dose test as the gold standard. The high-dose ACTH test is still considered the gold standard because, despite of its limitations, it has been the most frequently used test to diagnose CIRCI in critical care patients. In addition it has showed the most accurate relationship with morbidity and mortality. Nevertheless currently consensus is that it shouldn´t be used to guide the decision about steroid supplementation in septic shock (Dillinger et al., 2012).

Other tests for diagnosing CIRCI

Several other tests have been proposed to improve the diagnosis of CIRCI. These include the measurement of salivary cortisol, the response to CRH stimulation (Schuster, Macleod, Fernandez, Kumar & Barquist, 2012), the measurement of ACTH-dependent steroids such as dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEA-S) (Sayyed Kassem, El Sibai, Chaiban, Abdelmannan & Arafah, 2012), the measurement of cortisol/ACTH ratio (de Jong et al., 2015) and the measurement of the GCRs (Bhatia et al., 2014).

In *etomidate-treated patients* the use of cortisol/ACTH ratio and of the cortisol/ 11β-deoxycortisol ratio has also been used (Molenaar et al., 2012; Den Brinker et al., 2005). The latter is because etomidate inhibits the activity of the enzyme 11 β-hidroxilase, which converts 11β-deoxycortisol into cortisol (Wagner et al., 1984). Furthermore, a recent study also demonstrated that the determination of basal total cortisolemia remains a reliable criterion for the diagnosis of CIRCI in situations where etomidate has been administered (Asehoune et al., 2012).

The measurement of salivary cortisol levels have also been proposed because around 85% of salivary cortisol is available as free cortisol. In addition, their concentrations are strictly correlated with serum cortisol. In fact, increases in serum cortisol are reflected by increases in salivary cortisol by a lag of time of just 2-3 minutes (Moraes et al., 2011), and thus the latter can be used as a surrogate for serum free cortisol levels (Arafah, 2006). Studies performed in critically ill patients also demonstrated that the measurement of salivary cortisol is practical, simple to obtain and correlates well with serum free cortisol levels (Arafah, Nishiyama, Tlaygeh & Hejal, 2007). The limitations of the test are that in some patients it could be difficult to obtain saliva in enough quantity and quality to perform the test (e.g. ventilated patients) and the lack of standardization for saliva sampling. In addition, conditions

such as anemia, oral bleeding, oral candidiasis, and the use of chlorhexidine (oral decontamination) can interfere with cortisol determination (Moraes et al., 2011). More trials are necessary to validate the levels of salivary cortisol for CIRCI diagnosis.

The response to CRH stimulation has also been used to evaluate the integrity of the HPA axis in critically ill patients (Schuster et al., 2012). It was shown that the plasma cortisol response to a single CRH stimulation was impaired in nonsurvivors and that ACTH responsiveness was increased. Further studies are needed before this test becomes implemented in clinical patients.

The measurement of interstitial cortisol levels through the use of a microdyalisis probe inserted in the subcutaneous adipose tissue as also been suggested to provide an assessment of cortisol availability for target tissues (Vassiliadi et al., 2013; Venkatesh et al., 2010). However there are some inherent limitations associated to this technique which still need to be overcome before it can be used in clinical patients. First, edema is frequently present in critically ill patients. Second regional blood flow can be highly variable in critically ill patients. These factors have the potential to interfere with interstitial cortisol assessment. In addition, it is unclear if the values of interstitial cortisol in the subcutaneous tissue are a good reflection of cortisol levels of other tissues (Trzeciak et al., 2007).

Finally sophisticated techniques such genetic transcriptional surveys have been suggested to evaluate glucocorticoid tissue activity (Cohen & Venkatesh, 2009). To date these techniques remains largely investigational.

Diagnosis of CIRCI in HS

In the few studies where HS-associated CIRCI was assessed, the diagnosis was made by finding a level of total cortisol levels lower than 10 µg/dL (Stein et al., 2013) and of 25 µg/dL (Rushing et al., 2006) of by measuring the levels of total cortisol with the high-dose ACTH test (Cotton et al., 2008; Hoen et al., 2002).

2.11.8. Treatment of CIRCI

The treatment of CIRCI remains controversial, especially regarding the best time to start steroid therapy. In addition, the dose of supplementation is also highly debated. In the original randomized controlled trial of Annane et al., (2002), 200 mg of hydrocortisone and 50 µg of fludrocortisone were given daily for 7 days to treat patients in septic shock (Annane et al., 2002). The treated group showed a reduced need for vasopressor therapy when compared with placebo, although they did not displayed decreased mortality. A subgroup

analysis revealed that patients who displayed a cortisol increase lower than 9 µg/dL after ACTH stimulation and which had steroid supplementation had a 10% decrease in 28 day-mortality. The results of this study have been questioned because some of the patients had received etomidate, which could have introduced bias to the study´s result. A subsequent randomized-controlled trial, using the same dose of hydrocortisone for 5 days followed by a period of tapering did not find any benefit in survival, in both ACTH responders and non-responders (Sprung et al., 2008). Treated patients had indeed a higher blood pressure. Unfortunately they also t had a higher degree of sepsis relapse. This randomized controlled trial was also criticized because it was stopped prematurely and this could have reduced the study´s statistical power. The decision to stop prematurely the study was slow patient recruitment . A more recent systematic review of all available randomized-controlled trials has concluded that hydrocortisone therapy does not reduce mortality in severe sepsis (Patel & Balk, 2012).

In the most recent Surviving Sepsis Campaign guidelines, hydrocortisone therapy is only advised, with a weak level of evidence, to patients with hypotension refractory to vasopressor therapyand fluid resuscitation. It is not advised to patients with sepsis without septic shock (Dellinger et al., 2012). The guidelines state that hydrocortisone should be given in a dose of 200 mg by continuous intravenous (IV) infusion, followed by a quick tapering once blood pressure is maintained without the need of vasopressors.

The evidence that supports the use of steroid therapy for treatment of HS-associated CIRCI is even more limited than for septic shock. Most of evidence comes from experimental studies, which have been carried out since the decade of 70s of the last century. Proposed benefits of glucocorticoids in HS include prevention of myocardial depression (Merin, Eimerl, Raz, Tzivoni & Gotsman, 1978), decreased cytokine levels (Gundersen et al., 2003), attenuation of lipid peroxidation in reperfusion injury (Xia et al., 2003), modulation of NF-kB and HSP70 expression (Bini, Olivero, Trombetta, Castagna & Cotogni, 2008), preservation of liver and kidney metabolic activity, stabilization of lysosomal activity and better maintenance of blood and pulse pressure (Engerlbrecht, Mattheyse & Mouton, 1985). These studies revealed that the vascular effects seem to be more due to increased vessel sensitivity to α1-adrenoreceptor stimulation, than a direct glucocorticoid activity (Hoen et al., 2005). Despite this evidence, there is still no consensus regarding the use of steroid therapy in HS (Nelson et al., 2015; Stein et al., 2013; Rushing et al., 2006; Gannon et al., 2006).

The use of steroid therapy to counteract etomidate´s adrenal suppressive properties is even more controversial. The subject has been evaluated in several studies. A study in septic shock demonstrated that hydrocortisone administration did not prevent the increase in mortality associated to etomidate (Cuthbertson et al., 2009). In non-septic critically ill patients treated with etomidate, Payen et al. (2012) also did not report any benefits associated to

hydrocortisone administration (Payen et al., 2012). By contrast the use of etomidate associated to hydrocortisone in septic shock patients was associated to a decrease in 28-day mortality rates (Jung et al., 2012). Thus at the moment the benefits of adding hydrocortisone to etomidate to prevent etomidate´s associated CIRCI are unclear.

2.12. HS experimental models

Most of the knowledge regarding the pathophysiology of HS has been obtained through experimental models (Calzia et al., 2012; Hauser, 2005; Lomas-Niera et al., 2005). As Swanson et al., (2004, p.866) wrote, “biomedical models are determined as surrogates for a Human biologic system, that researchers use to understand physiological and pathological functions of the human body and to provide a basis to therapeutic intervention in diseases”. In HS research, several experimental models have been developed with different aims and characteristics. Each one has its own and specific advantages and disadvantages (Fülöp, Turóczi, Garbaisz, Harsányi & Szijártó, 2013). HS models differ in the specie, age and gender of the animal which is used, if the animal is conscious or under general anesthesia, in the way how HS is induced, duration of HS, type of resuscitation, and if they combine HS with other comorbidities (e.g. head trauma, limb fracture).
In general small animal models are used to investigate the pathophysiological mechanisms of HS whereas large animal models are used to test preclinical diagnostic and therapeutic approaches. In this part we will focus more in HS models which rely in the rat (*Rattus norvegicus*).

The rat as a model of HS

Rats are commonly used in HS experimental research due to several advantages. These include its availability, decreased genetic variability (most are inbred strains), easy of care, cost and ethical acceptability. Models using rats have also a high degree of reproducibility. When compared with mice their larger size make it easier to induce HS and their larger blood volume permits to obtain samples with larger volumes. There are also many reagents available to evaluate the genetic, immunological and endocrine response in rats. The rat´s immune response to HS seems also to be similar to Humans (Hauser, 2005),
They also have disadvantages. The first is that their genetic similarity to Humans is only of 89-90% (Lomas-Niera et al., 2005; Gibbs et al., 2004). Another disadvantage is that there are fewer reagents available when compared to mice. There are also less available knockout (KO) or transgenic strains, which can increase variability in data interpretation. In addition

when using rats, one also must take in account the gender, age and strain as these can also become a source of variability.

Types of HS models

Currently there are three types of HS-models: *pressure-fixed model*; *volume-fixed model* and *uncontrolled hemorrhage*. These will be described in the following sections.

Pressure-fixed model

In this model animals are bled to a predetermined blood pressure and once this is achieved, animals are maintained in that blood pressure for a limited amount of time through repeated bleeding or fluid administration, as necessary (Fülöp et al., 2013). The main advantage of this model is that the degree and duration of hypotension is accurately controlled through blood pressure monitoring, which greatly increases reproducibility of the model. In addition it permits to evaluate physiological mechanisms and organ injuries after HS depending on the intensity of hypotension (Lomas-Niera et al., 2005).
The main disadvantage of this method is that it does not resemble the clinical situation (Tsukamoto & Pape, 2009).
The pressure-fixed model was first described in the first half of the XX Century (Wiggers, 1942; Penfield, 1919). Since then, it has been used to evaluate the effects of HS in the inflammatory response, cerebral, lung and liver injury and the effects of many resuscitation strategies on organ function and outcome (Lomas-Niera et al., 2005). There is currently no agreement on which should be the target blood pressure and the ideal length of shock required to set up a reliable HS pressure-fixed model. Many variations of the model exist, with target blood pressures ranging from 20-55 mmHg and duration of the HS state from 15 min to 3 hours (Fülöp et al., 2013). In the particular case of the rat, studies have reported a target blood pressure of 25-50 mmHg and duration of HS from 15 to 180 min (Cai et al., 2011; Rupani et al., 2007; Yang et al., 2006; Hoppen et al., 2005; Gonzalez et al., 2003; Zingarelli et al., 1997).

Volume-fixed model

In this model, a predetermined percentage of the total blood volume is removed in a fixed amount of time to induce HS. Total blood volume is normally estimated based in the animal´s weight. In rats, blood volume is estimated to be between 54 to 70 ml/kg (Rushing et al., 2006; Diehl et al., 2001). After HS is induced, animals are allowed to recover and return to

the cage or are resuscitated with different resuscitation regimes. The main advantage of this method is that permits to study the body´s compensatory mechanisms associated to a fixed amount of blood loss. However the degree of hypotension achieved is variable, which limits the experiment´s standardization and reproducibility. In addition, in rats with weight between 100-400 gr, the body weight influences the amount of blood that can be collected. In fact the ratio blood volume/body weight decreases with increasing body weight, because larger animals have more fat and in proportion less blood volume. This leads to differences in the amount of blood which is collected, leading to significant bias in data interpretation. The model also does not reproduce accurately the real clinical situation.

Despite these limitations, the volume-fixed model is widely used to evaluate the pathophysiology of carbohydrate metabolism, anaerobic glycolysis and lactacidemia, histopathological abnormalities and efficacy of several therapeutic measures (Fülöp et al., 2013). In addition because the Advanced Life Trauma Support guidelines state that a bleeding exceeding 40% of blood volume is associated to more than 30 % of mortality (Frankel et al., 2007), most investigators use the fixed-volume model to achieve this percentage. In rats a blood loss of 40% of blood volume is normally achieved through collection of a fixed volume of 45% of the body weight, in 10 to 15 minutes. Models where30 to 60% of blood volume are collected have also been reported in the literature (Johnson et al., 2010; Troy et al., 2003; Diehl et al., 2001; Santibanez-Gallerani et al., 2000).

Frankel et al., (2007) refined the fixed-volume model by dividing hemorrhage in two periods, a first characterized by a rapid rate of hemorrhage followed by a second period at a slower rate (Frankel et al., 2007). By inducing hemorrhage through this way, the authors reported an increased physiological response (heart rate, serum lactate, and volume of fluid resuscitation) as compared with a constant rate of hemorrhage (conventional manner).This approach resulted in a more physiological and consequently in a more accurate model of the real clinical scenario. This model might be the most improved model of volume-fixed hemorrhage so far.

Uncontrolled hemorrhage

In the uncontrolled hemorrhage model, HS is induced by a standardized vascular trauma (crush/laceration of liver or spleen, amputation of an extremity, artery injury). Although it is more difficult to reproduce, this method is the one which more closely resembles the real clinical scenario. This is because HS management is more dependent of the animal´s own hemostatic and cardiovascular compensatory mechanisms (Fülöp et al., 2013). The uncontrolled hemorrhage mechanism has been mainly applied to evaluate therapeutic approaches.

In the last decade, several refinements have been performed to this model, permitting the evaluation of different fluid regimes and other therapeutic approaches including hypothermia or hemostatic agents (Heinius, Hahn & Sonden, 2011; Krausz & Hirsh, 2003; Burris et al., 1999). In rats, uncontrolled hemorrhage models have been reported where HS was induced by artery injury, splenic (Krausz & Hirsh, 2003) and liver injury (Matsuoka, Hildreth & Wisner, 1995) and tail amputation (Kentner et al., 2007).

Other models

In an effort to improve the knowledge of HS and to increase model´s capacity to mimic the real clinical situation, several models have been developed that combine HS with other injuries, including fractures, cerebral, lung and other organ´s injuries. These have been essential to augment our knowledge regarding the influence of the injury itself in the pathophysiology of critical ilness following trauma and HS (Fülöp et al., 2013).

Confounding variables

When one chooses a type of HS model we must take in account some variables which have the possibility to influence data collection and interpretation. Several studies have demonstrated that host factors such as strain (Klemcke et al., 2011), age and sex (Mees et al., 2008) influence the response to HS.

Different rat strains have different neuroendocrine responses determining its susceptibility to auto-immune and inflammatory diseases (Joe, Garret, Dene, Remmers & Meng, 2003; Tonelli, Webster, Rapp & Sternberg, 2001). In addition, differences between survival times and percentage of survival exhibited by different rat strains are known to be heritable and quantitative traits (Klemcke et al., 2011). The genetic and epigenetic mechanisms which are responsible for these traits are under active investigation (Klemcke et al., 2011).

Regarding gender, it is well known that female animals have a survival advantage compared to males, when submitted to trauma, HS and sepsis (Klemcke et al., 2011; Diodato Knoferl, Schwacha, Bland & Chaudry, 2001). Although the nature of this protective mechanism is still incompletely understood, it is believed that it may be related with the presence of female hormones, which are known to play a pivotal role in maintaining immune function (Angele, Schwacha, Ayala & Chaudry, 2000).

Age also contributes significantly to the response to HS. In fact, it has been demonstrated that older animals have depressed immune function and increased organ damage following HS (Mees et al., 2008).

In most HS models, animals are submitted to general anesthesia. However it is known that general anesthesia depresses respiratory function and metabolic demand (Brunner, Cheng & Berman, 1975), moderates CNS function (Hauser, Dayao & Zukowskagrojec, 1995) and decreases the normal compensatory cardiovascular mechanisms (Stenseth et al., 1993). In addition, several anesthetics affect immune cell function (Kelbel & Weiss, 1997). For instances, in many experiments, rodents are anesthetized with volatile anesthetics, which possess immunomodulating properties (Bedirli et al., 2012).

Besides, in some experiments, general anesthesia is associated to mechanical ventilation which is associated to inflammatory responses (Van Wessem, Hennus,Heeres, Koenderman & Leenen, 2013).Interestingly, the inflammatory response induced by mechanical ventilation can itself be modulated by the choice of the anesthetic (Mahmoud & Ammar, 2011; Schilling et al., 2011). To circumvent these limitations several HS are currently available models which use conscious animals and limit the use of general anesthesia to a minimum (normally to perform intravascular placement).

Finally HS models may also include the use of analgesics. These can be confounding factor as well and this theme will be more developed in the following sections.

Opioid analgesia and HS models

As described previously, HS research depends considerably from the use of experimental models. Some of these experiments involve complex and invasive procedures which can be itself a considerable source of pain. However, in many situations the use of analgesics in HS research is withheld. There are several reasons for this attitude. One is that the use of analgesics increases the cost, time and personnel resources. In addition, in the case of opioids, as they are controlled substances, its administration is associated to security and reporting concerns (Hugunin, Fry, Shuster & Nemzek, 2010). Furthermore, analgesic drugs, and in particular opioids, are known to influence immune status. Common examples of these are morphine and fentanyl (Al-Mousawi et al., 2010; Molina et al., 2006; Vallejo, de Leon-Casasola & Benyamin, 2004; Hilburger et al., 1997). As a consequence the use of opioids in the perioperative period is discouraged to avoid bias in the interpretation of immune variables.

Unfortunately, the practice of withholding analgesia is incorrect for several reasons. It is well known that pain caused by surgery and trauma can interfere with immunological and endocrine functions (Hugunin et al., 2010). In addition, in the real clinical scenario, the use of analgesics for severe trauma is viewed not only as a humanitarian gesture, but also an indispensable therapeutic maneuver (Hedderich & Ness, 1999). Thus, one may say that the practice of withholding pain management in experimental models of HS is not only unethical

but also contributes to decreases the value of the experiment in modelling the real clinical situation. It is also associated to legal concerns because current EU regulation (2010/63/EU Directive) regarding laboratory animal experimentation emphasizes the use of procedures that limit or abolish the induction of pain in experimental animals.

Immune effects of opioid drugs

The immune effects of opioid drugs have been well recognized (Al-Hashimi, Scott, Thompson & Lambert, 2013; Molina et al., 2006; Gomez-Flores & Weber 2000), although the potential sites/mechanism (s) for this modulation are still incompletely understood. It has long been assumed that opioid-induced immune modulation occurs via a combination of direct actions on the immune cell itself, via the HPA and sympathetic nervous system axis, or through central mechanisms (Molina et al., 2006; Gomez-Flores & Weber 2000).

Regarding direct immune effects, if classical opioid receptors are present on the surface of immune cells is controversial (Pomorska, Gach & Janecka, 2014; Al-Hashimi et al., 2013). The evidence for HPA activation as a mechanism of opioid-induced immunomodulation is also poor and is species-dependent (Al-Hashimi et al., 2013).

Part of our incomplete understanding regarding the role of opioids in immune function reflects the circumstances in which data about this subject was obtained. In effect, the reported immune effects varied accordingly experimental conditions, including specie, cell type, nature of the immune stimulus, the receptor which the drug preferentially ligates, drug type, dosage and route of administration.

At a cellular level, it has been demonstrated that opioid drugs change several intra-cellular signaling pathways through ligation to opioid receptors (Chen, Law & Loh, 2006) or indirectly, through the modulation of other substance´s release such as substance P (Wang et al. 2004), and NO (Wypasek, Natorska, Mazur & Kolaczkowska, 2012). The activation of these intracellular pathways leads to modulation of several transcription factors including NF-κB, Sp1, STAT3, AP-1, IRFs and cAMP-response element-binding protein, and subsequently to changes in gene expression (Mosser & Zang, 2008). Opioid modulation of NF-κB, one of the most important transcription factors in immune cells (Li & Verma, 2002) has been particularly studied. μ agonists activate or repress NF-κB activation in a cell-specific, time- and concentration-dependent manner (Finley, Happel, Kaminsky & Rogers, 2008; Bidlack et al., 2006; Chen, Law & Loh, 2006) whereas κ agonists inhibit NF-κB activation (Chen, Law & Loh, 2006). Interestingly it has also been demonstrated that the binding of different molecules to the same opioid receptors can also activate different intra-signaling pathways, leading to different cellular responses. This has been demonstrated with buprenorphine and

morphine, which regulate G-protein coupling to the μ receptor differently (Saidak et al., 2006).

Buprenorphine

Buprenorphine is an orivapine derivative opioid, 25 to 40 times more potent than morphine which has a complex pharmacology. Buprenorphine was originally described as a partial μ agonist, but it also has agonistic activity in the nociceptine opioid peptide (NOP) receptor, antagonist activity at the κ and ε receptors and both actions at the δ receptor (Lewis & Husbands, 2004; Lutfy & Cowan, 2004). In addition, it has been demonstrated that its 3 major metabolites, norbuprenorphine, buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide are metabolically active and have distinct biological activity from the parenteral drug probably due to different receptor affinity (Brown et al., 2011).

Buprenorphine has been available for several decades and was traditionally used as an alternative to methadone in heroin addicts. In recent years it has become increasingly popular for the treatment of several pain states and it was even suggested for the treatment of refractory depression and cocaine addiction (Lutfy et al., 2003). It is also the most commonly used analgesic for pain control in laboratory rodents (Stokes, Flecknell & Richardson, 2009) and several studies have documented its effective analgesic effects in the peri-operative period for these animals (Curtin et al., 2009; McKeon et al., 2011).

When compared with other opioids, in particular μ agonists such as morphine and fentanyl, buprenorphine has several therapeutic advantages. It is considered a safer drug because respiratory depression only occurs at high doses. In addition, due to its longer half-life, the withdrawal symptoms are milder and there is less risk of drug dependence (Brown et al., 2011). It is also believed that buprenorphine is not immunosuppressive such as morphine and fentanyl and that it does not adversely affect the HPA axis (Davis et al., 2012).

The literature supports the concept that buprenorphine or does not affect or actually decreases HPA axis activity in rats (Goldhkuhl et al., 2010; Goldhkuhl et al., 2008; Gomez-Flores et al., 2000). In non-operated rats, the administration of buprenorphine did not changed CS levels (Pechnick et al., 1985). In addition, several experimental models which used buprenorphine to provide peri-operative analgesia reported a decrease in CS levels in the post-operative period (Goldhkuhl et al., 2010; Goldhkuhlet al. 2008; Franchi, Panerai & Sacerdote, 2007).

The notion that buprenorphine is not immunosuppressive in rats has been suggested by the several studies (Martucci, Panerai & Sacerdote, 2004; D`Elia, Patenaude, Hamelin, Garrel & Bernier, 2003; Gomez-Flores & Weber 2000). However other studies, with different experimental methodologies, suggested the opposite. Piersma, Daemen, Bogaard &

Buurman, (1999) demonstrated that buprenorphine decreases TNF-α production in response to LPS administration in rats and Carrigan, Saurer, Ijames & Lysle, (2004), showed that buprenorphine suppresses splenic NK cell activity, lymphocyte proliferation and IFN-γ production in a dose-dependent manner (Carrigan et al., 2004; Piersma et al., 1999) . Finally, Cotroneo et al., 2012 in the cecal ligation puncture (CLP) model of sepsis, showed that buprenorphine decreased peripheral neutrophil counts and survival in male mice in a dose-dependent manner, suggesting that some of buprenorphine´s immunopathological effects of the drug are gender specific. Other studies demonstrated that buprenorphine significantly reduced (Sacerdote, 2008) and even prevented surgery-induced immunosuppression (Franchi, Panerai & Sacerdote, 2007) and metabolic stress (Goldkuhl et al., 2006). Some authors actually suggested that the analgesic has an immunostimulatory influence (Van Loveren et al., 1994).

There are no reports describing the use of buprenorphine as analgesic in rats submitted to HS. However buprenorphine has been used for this purpose in models of endotoxic shock (Tseng & Tso, 1993), sepsis (Cotroneo et al., 2012; Donaldson, Vesey, Wilks & Hinds, 1988) and burns (Al-Mousawi et al., 2010). In these studies buprenorphine was able to modulate several immunological variables although the significance of these findings is currently unknown.

3. Methods

All experiments were performed in accordance with the ethical standards of Faculdade de Medicina Veterinária da Universidade de Lisboa, in compliance with the Portuguese legislation for the use of animals for experimental purposes (Decreto-Lei nº 129/92 and Portaria nº 1005/92, DR nº 245, série I-B, 4930-42) and with the European Union legislation (EU Directive 2010/63/EU).

3.1. Experimental animals

Twelve-week-old male Wistar rats (*Rattus norvegicus*) (Charles Rivers, Barcelona, Spain), weighing 250-450 grams, were used in this study. We only used male rats because in this gender, HS is associated to a higher degree of organ damage (Mees et al., 2008). Animals were housed (n = 3 per cage) in a climate-controlled room under standard conditions (20-24ºC; 12 hr light/dark cycle). Water was provided *ad libitum* and food consisted of rat chow (Harlan, 2014). All animals were acclimatized for seven days before the experiments, to minimize stress.

3.2. Study groups

Rats were randomly and blindly allocated to one of four body weight-matched groups: G0 (n=16), G1 (n=16), G2 (n=16) and G3 (n=16). Randomization was made after attributing a piece of paper with a number to each animal. The animals were than allocated to each group in rotation based in a computer-based random number generator. Rats from G0 (the control group) were submitted to general anesthesia, mechanical ventilation and surgical intervention. Rats from G1 had the same procedures than G0, but also received buprenorphine. Rats from G2 had the same procedures than G1 but were submitted to HS. Rats from G3 were treated exactly like G2, except that they received etomidate.

3.3. *Experimental procedures*

Figure 9 displays the experimental protocol. A more detailed explanation of the experimental procedures is described in the following sections.

Figure 9**:** Experimental time line.

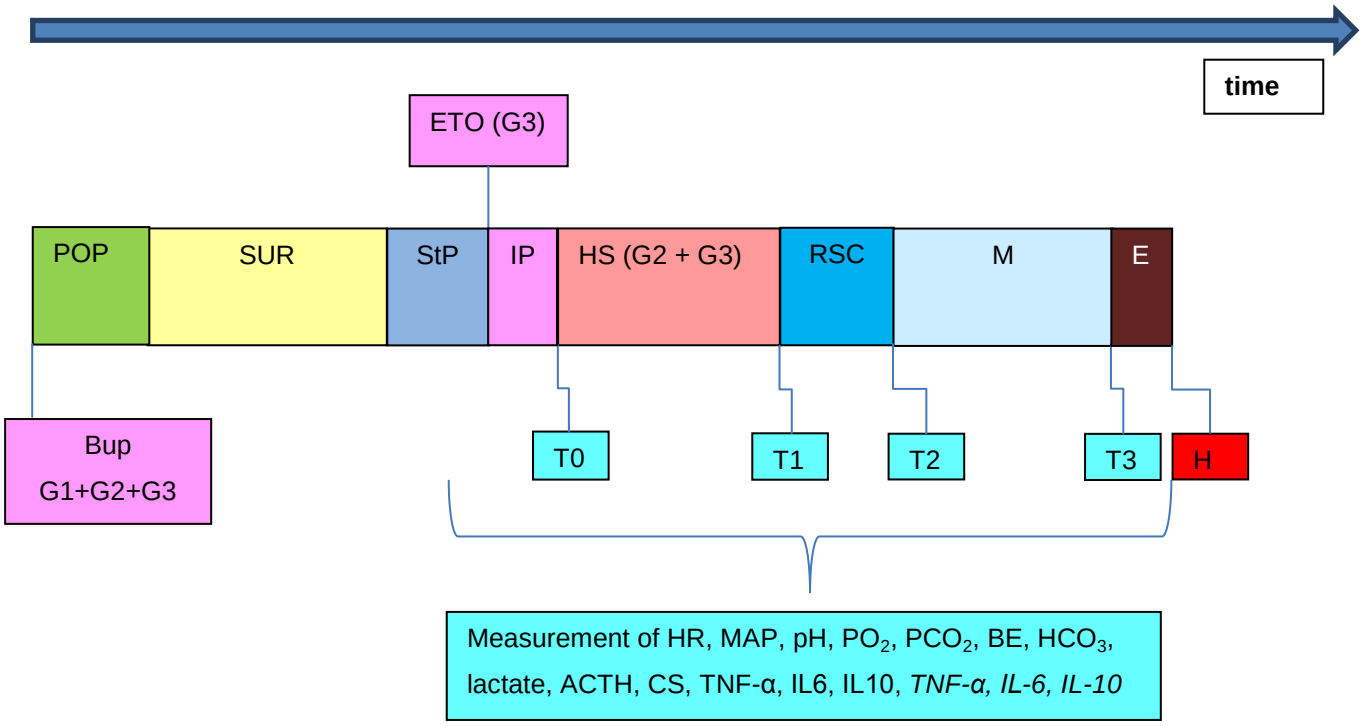

Figure 9: Experimental time line.G0: control animals with general anesthesia, mechanical ventilation; G1: similar to G0 but with buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. POP-preoperative period; SUR, surgical intervention; StP, stabilization period; IP, intermediate period (in G3 only, lasting 5 minutes); HS, hemorrhagic shock; RSC, resuscitation; M, maintenance stage; E, euthanasia; HP-histopathological analysis; Bup, buprenorphine; ETO, etomidate; ACTH, adrenocorticotropic hormone; CS corticosterone; TNF-α, tumor necrosis factor-α; IL6, Interleukin 6; IL10, interleukin 10; *TNF-α*, circulating levels of mRNA of *TNF-α*; *IL6*, circulating levels of mRNA of *IL6*; *IL10*, circulating levels of mRNA of *IL10*. ETO was only administered in G3. In G1, after StP, the animals went directly to M until E because they did not had the HS and RSC stages.

3.3.1. Anesthesia and surgical procedure.

Rats from all experimental groups except G0 were pre-medicated with 0.05 mg/kg buprenorphine (Budale®, Dechra, UK) to induce preemptive analgesia. Buprenorphine was injected subcutaneously (SC), 20 minutes prior induction of general anesthesia. General anesthesia was initiated by placing the animals in an induction chamber (World Precision Instruments, UK, Europe) previously saturated with 100% oxygen and 5% isoflurane (IsoFLo®, Abbott, USA). Once anesthetized, rats were moved and placed in dorsal

recumbency over a water-based heated pad. Isoflurane anesthesia was maintained through face mask until a tracheostomy tube was placed. ECG was registered continuously through lead wire probes (ECG; ML136 Animal Bio Amp, ADInstruments, UK). A respiratory sensor was placed over the thoracic wall to measure respiratory rate. A rectal probe (MLT1403, ADInstruments, UK) and an oximetry tail sensor (ADInstruments, UK,) were placed to record continuously rectal temperature (kept between 35 and 38°C) and pulse oximetry, respectively. The pedal withdrawal reflex was used to help in assessing the depth of anesthesia. The anesthetic plane was considered adequate if a toe pinch did not induce the withdrawal reflex. An intravenous catheter (Introcan®, 26 Gauge, B. Braun Medical, Portugal) was placed in the left femoral vein for fluid and drug administration. Another catheter was placed in the right external carotid artery and connected to a three-way stopcock for blood sampling and arterial blood pressure measurement. The carotid catheter and the three-way stopcock were connected to a fluid-filled pressure transducer (MLT844, ADInstruments, UK) which was connected to blood pressure amplifiers (ML221 Bridge Amp, ADInstruments, UK). Arterial blood pressure [systolic, diastolic and MAP], ECG, rectal temperature, pulse oximetry and respiratory frequency data was transmitted to a data acquisition unit (PowerLab®, ADInstruments, UK) where it was analyzed by a specific software (LabChart Pro®, ADInstruments, UK).

After placement of the carotid catheter, a tracheostomy tube was placed. Volume-controlled ventilation was initiated with a small animal ventilator (CWE Small Animal Ventilator, World Precision Instruments, UK). General anesthesia was maintained with isoflurane at concentrations of 1.5-2%, administered through the tracheostomy tube. Oxygen was also delivered through the tracheostomy tube and the inspiratory fraction of O_2 (FiO_2) was maintained at 100% until the end of the experiment. Initial ventilator settings consisted in a tidal volume of 10 ml/kg and respiratory rate of 110-125 breaths/min. These parameters were adjusted as the experiment progressed, accordingly to results of arterial blood gas analysis, in order to maintain normocapnia (35-45 mmHg) and normoxemia (PO_2 >85 mmHg). Once surgical instrumentation was completed, a stabilization period was allowed before inducing HS in G2 and G3. This lasted 10 minutes and was followed by the intermediate period in G3. In G2 the stabilization period lasted 15 minutes. This period permitted the recovery from the hemodynamic instability caused by initiation of mechanical ventilation. At T3 and after blood sampling, euthanasia was performed by pentobarbital administration (Eutasil®, Ceva, Portugal) at 100-150 $mg.kg^{-1}$ through the femoral catheter.

Figure 10 illustrates one of the animals of G2, during the experimental procedure and its corresponding monitoring parameters displayed in the data acquisition unit.

Figure 10: Experimental procedure-surgery and monitoring

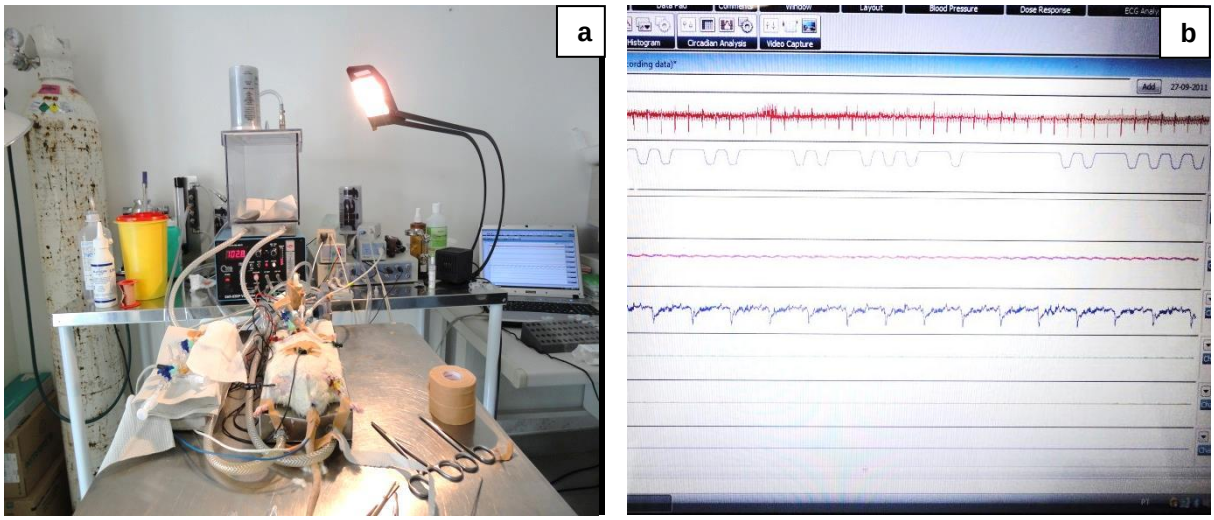

Figure 10: **a)** animal of G2, in the stabilization period, immediately before inducing HS; **b)** monitoring parameters correspondent to the animal in **a)** displayed in the data acquisition unit.

3.3.2. Sample collection and anesthesia monitoring.

At four time points (T0, T1, T2 and T3), a sample of blood (500 µl) was collected (see Figure 11). To prevent activation of the HPA axis following blood collection, after each sampling, 1 ml of normal saline was administered IV, except at T0 in G2 and G3, when blood collection coincided with HS induction. At T0, T1, T2 and T3, MAP, heart rate (HR), temperature, ECG, ventilator parameters and depth of anesthesia were recorded. T0, the initial time point, was set at the end of the stabilization period T1, T2, and T3 were set at 90, 150 and 240 minutes post T0, respectively. HR and MAP collected at each time point were used for analysis and constituted the hemodynamic variables.

Immunological and hormonal variables.

At all time points, plasma levels of TNF-α, IL6, IL10, ACTH and CS were determined. To obtain plasma, blood samples were collected into 200 µl sterile heparin-coated tubes (FactorMed, Portugal) followed by centrifugation at 12000 rpm for 15 minutes. Plasma was then stored at -20°C until further analysis. ACTH, CS (rat stress hormone panel Millipore, Arium Laboratórios, Portugal) TNF-α, IL6 and IL10 (Rat Cytokine/Chemokine, Millipore, Arium Laboratórios, Portugal) plasma levels were determined by Multiplex/Luminex technology, as described by others (Vignali, 2000).

Figure 11: Example of blood sampling and analysis in the I-STAT machine

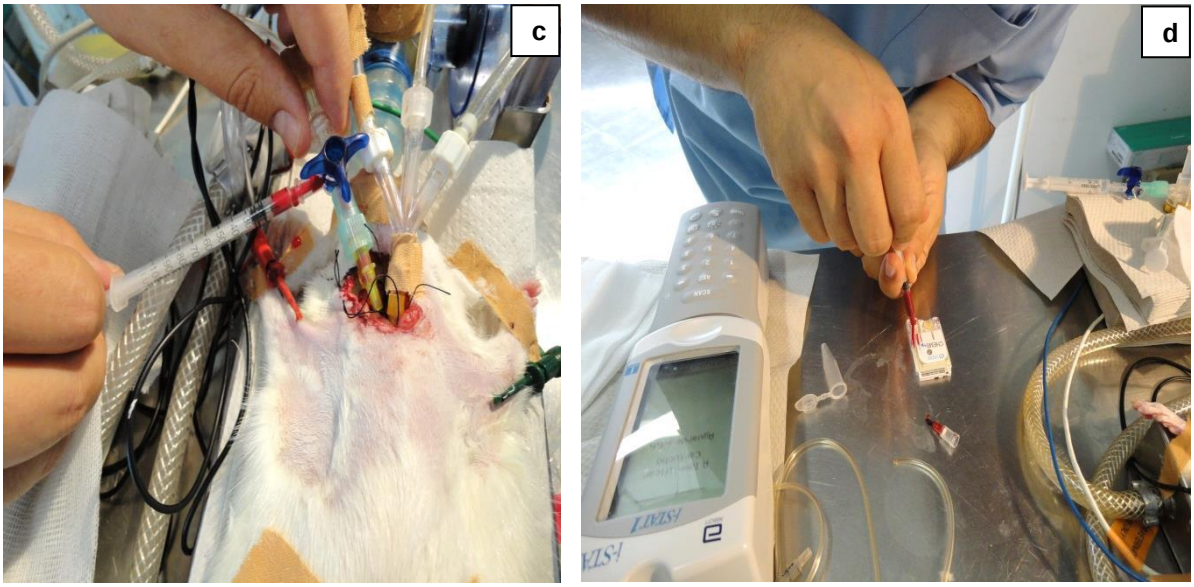

Figure 11: **c)** blood sampling in the same animal of **a)** and **b)**; **d)** analysis of the correspondent analysis in the I-STAT machine

Metabolic variables.

At all time points, 100 µl of blood was used to measure metabolic variables, including pH, arterial pressure of O_2 (PO_2), arterial pressure of CO_2 (PCO_2), BE, HCO_3 and lactate, through a portable blood gas analyzer (I-STAT Analyzer 300, I-STAT Corporation, Abbot, USA). The arterial pressure of O_2/Inspiratory Fraction of O_2 (PO_2/FiO_2) ratio was calculated as indicator of lung dysfunction.

Molecular Biology Variables: sample collection, mRNA extraction and complementary deoxyribonucleic acid (cDNA) synthesis.

At all time points, the correspondent mRNA of *TNF-α*, *IL6*, *IL10* and *β-actin* genes (*β-actin* was chosen as the *reference* gene) was determined in blood. For this, 200 µl of blood samples were collected into tubes containing RNA stabilizer specific for RNA extraction in animal blood (Qiagen, Hilden, Germany). After collection, samples were refrigerated at 4°C for 24 hr until further processing. RNA was extracted from blood using the "Q-Amp RNA blood mini kit'' (Qiagen), according to the manufacturer's instructions. Once obtained, it was dissolved in diethylpyrocarbonate-treated water and stored at -70°C until further analysis. DNA digestion was performed with the RNase-free DNase Set (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically at 260 and 280 nm. cDNA was produced by using the "Transcriptor High Fidelity CDNA Synthesis Kit" (Roche, Portugal) as described by others (Gil, Sepúlveda,

Albina, Leitão & Martins, 2008). Primer pairs for *β-actin*, *TNF-α*, *IL6* and *IL10* genes were designed from various exons using specific murine sequences obtained from the GenBank database, as shown in Table 1

Table 1: Primer sequences for *IL6*, *TNFα*, *IL10* and *β-actin* used for RT-PCR and obtained from the GenBank database.

IL6 Forward	GGAAATGAGAAAAGAGGAAATTTGC
IL6 Reverse	TGACTCTGGCTTTGTCTTTCTTGT
TNF-α Forward	GAACTCCAGGCGGTGTCTGT
TNF-α Reverse	TGGGAACTTCTCCTCCTTGTTG
IL10 Forward	CCCTGGGAGAGAAGCTGAAGA
IL10 Reverse	CACTGCCTTGCTTTTATTCTCACA
β-actin Forward	GTGAAAAGATGACCCAGATCATGT
β-actin Reverse	CACAGCCTGGATGGCTACGT

Table 1. TNF-α: Tumor Necrosis Factor-α; IL6: Interleukin 6; IL10: Interleukin 10.

3.3.3. Relative quantification of TNF-α, IL6 IL10 and β-actin mRNA expression.

TNFα, *IL6, IL10* and *β-actin* mRNA was quantified by fluorescence-based quantitative real-time polymerase chain reaction (RT-PCR) according to MIQE guidelines (Bustin et al., 2009). Amplicons for *TNF-α*, *IL6, IL10* and *β-actin* were obtained by using an Applied Biosystems 7300 Real-Time PCR thermocycler machine (Perkin Elmer, Portugal) following the manufacturer's instructions (Gil et al., 2008). Data on relative mRNA quantification was analyzed using a real-time PCR Miner algorithm (Zhao & Fernald, 2005).

3.3.4. HS induction and resuscitation.

At T0, after blood sampling, HS was induced in G2 and G3 by collecting 30% of blood volume (estimated as 54 ml/kg) (Rushing et al., 2006) from the carotid artery (Figure 12). Blood collection was divided into two stages. In the first stage, the first 15% of blood volume was collected in 10 min. The remaining 15% was collected in the next 20 min. By dividing blood collection into two stages of different velocities, we aimed to induce a more natural model of hypovolemia, as it was proposed by some authors (Frankel et al., 2007). Because our goal was to induce a mild state of HS, hemorrhage was stopped when 30% of the estimated blood volume was removed and/or when MAP reached and stabilized at 45

mmHg. In this way, the survival rate of the animals at the end of the experiment was 100%. The difference between the estimated and the amount of blood which was actually collected to induce HS (Δvolume) was determined for each rat. The individual Δvolumes were used to calculate an average Δvolume for G2 and G3.

All blood which was not used to determine hormonal, immunologic, metabolic and molecular biology variables was collected into several 1 ml sterile aliquots previously filled with 0.12 ml of sodium citrate. Once filled, the aliquots were maintained in constant motion by using a laboratory agitator (LIC Instruments), until blood was used in the resuscitation phase. At T1, after blood sampling, resuscitation was initiated by administering the collected blood with normal saline in a 1:3 ratio, respectively. Both fluids were warmed at body temperature and administered through the femoral vein catheter with a syringe pump (Perfusor® fm (MFC), B. Braun Medical, Portugal) (Figure 12). The resuscitation phase lasted 30 minutes. Once resuscitation was completed, fluid therapy was maintained with warmed normal saline administered at maintenance rate (2 ml/kg/hr) until the end of the experiment.

Figure 12: Blood collection to induce HS and resuscitation

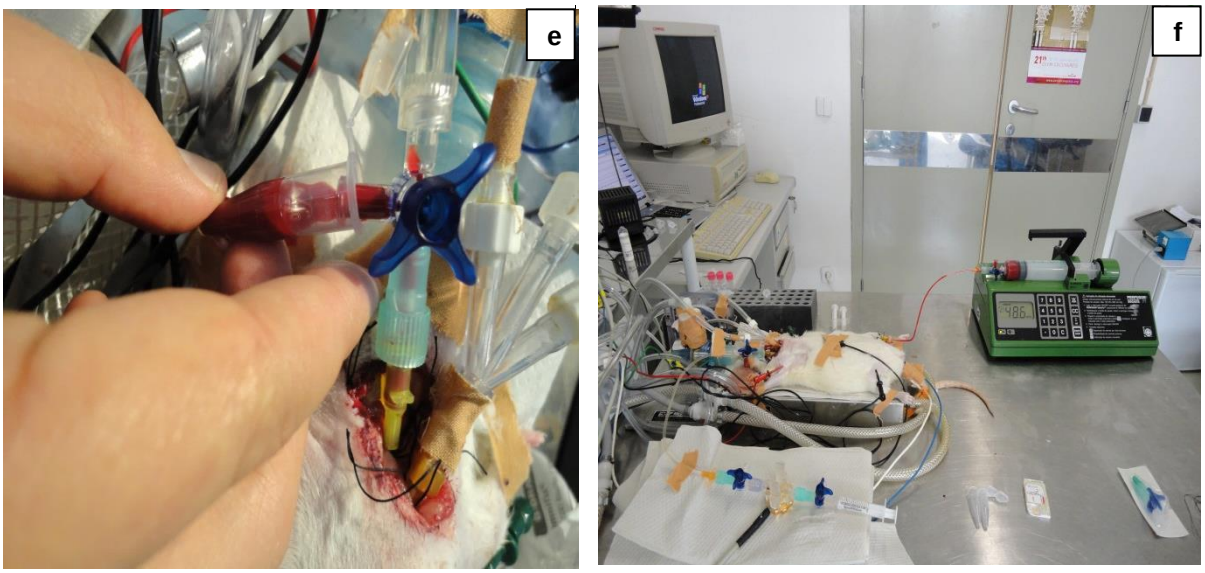

Figure 12: **e)** blood collection to aliquots previously filled with 0.12 ml of sodium citrate; **f)** resuscitation with normal saline and blood with the aid of a syringe pump.

3.3.5. Etomidate administration.

In rats from G3, 5 minutes before T0, etomidate (Etomidate®, Lipuro, 2 mg/ml, B. Braun Medical, Portugal) was administered by a single IV bolus at the dose of 1 mg/kg. This dose of etomidate is known to induce reliable hypnosis in rats (Cotten et al., 2009).The total volume of anesthetic solution was 0.5 ml/kg for each animal. In rats of G1 and G2, the same amount of normal saline was given instead of etomidate.

3.3.6. Histopathological variables.

Adrenal gland necrosis assessment. After euthanasia both adrenals were collected and placed in 1% formaldehyde. Several 10 µm-thick tissue sections were than obtained and stained with hematoxylin and eosin. Two slides of each adrenal gland were evaluated by a pathologist blinded to the study, which looked for changes such as tissue edema, lipid vacuolization, and inflammatory cell infiltration. The degree of adrenal necrosis was also quantified in a score which was adapted from another score, published by others (Rushing et al**.**, 2006). In our scoring system, necrosis was quantified in 5 categories depending on the percentage of gland affected. Thus, score 0 represented a complete absence of necrosis and scores 1, 2, 3 and 4 corresponded to a 25%, 25-50%, 50-75% and more than 75% of the gland being affected by necrosis, respectively. The score of necrosis was first determined in each of the two slides of the adrenal gland. This was used to determine the score of necrosis of each gland by calculating an average. By using the scores of the two glands to create an average, the score of each animal was determined. Finally using the same approach, the necrosis score of each group was determined by determining an average from the necrosis scores of all animals comprising each group.

TUNEL assay.

TUNEL staining was performed according to the manufacturer's instructions (ApopTag® Plus Peroxidase In Situ Apoptosis Kit, #S7101, Merck KGaA, Darmstadt, Germany). Tissue specimens were examined using an Axioskop bright-field microscope (Carl Zeiss GmbH, Gena, Germany). Tissue sections 10 µm thick were deparaffinized in three changes of xylene, and next hydrated with two changes of absolute ethanol, followed by three consecutive washes in 95%, in 70% ethanol, and in phosphate buffer saline (PBS), 5 min per wash. Then, samples were incubated with freshly diluted proteinase K (20 µg/ml) in PBS, for 15 min at room temperature, followed by two washes in ddH2O, 2 min, room temperature. Subsequently, samples were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After adding the equilibration buffer, sections were treated with terminal deoxynucleotidyltransferase for 60 min at 37ºC. Specimens were then incubated with anti-digoxigenin peroxidase conjugate for 30 min at 37ºC, stained with 3, 3'-diaminobenzidine substrate, and counterstained with 0.5% methyl green. Finally, slides were rinsed, dehydrated, and mounted. A negative control was prepared by omitting the TdT enzyme to control for non-specific incorporation of nucleotides or binding of enzyme-conjugate.
The slides were observed and photo documented using an Olympus BH-2 microscope. To quantify TUNEL-positive nuclei, a total of 12 high-power fields (x400) were randomly

examined for each animal, six from each adrenal gland. Three fields from the adrenal cortex and three from the adrenal medulla were evaluated. The apoptotic index (in percentage) was obtained from the ratio between the apoptotic and the total number of cells counted in each field, as described by others (Yu et al., 2012). The total number of apoptotic and non-apoptotic cells in each field was obtained with the aid of the Image J software (NIH, http://rsb.info.nih.gov/ij/), especially adapted for this purpose. This allowed the determination of an average cortical and medullary apoptotic index for each adrenal gland. Subsequently, by pooling the results of the two adrenal glands, the average cortical and medullary apoptotic indexes for each animal were determined. Finally, with the values of all animals belonging to the same group the average cortical and medullary apoptotic index for each group was calculated and compared through statistically methods.

Caspase-3 immunofluorescence microscopy analysis.

Because necrotic cells can also stain positive by TUNEL (Saraste, 1999), the presence of apoptosis was confirmed by determining the presence of active caspase-3, a protein which has an essential role in the apoptotic process (Porter & Jänicke, 1999). This approach had already been described by others (Yu et al., 2012). For this assessment, 10 µm thick paraffin-embedded adrenal sections were deparaffinized, rehydrated and boiled 3 times in 10 mM citrate buffer, pH 6. Sections were then incubated for 60 min in blocking buffer, containing 10% (v/v) normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) in PBS with 0.1% (v/v) Triton X-100 (Sigma-Aldrich), and subsequently in diluted primary antibodies overnight at 4 °C. After rinsing, the primary antibody was developed by incubating with DyLight 488- (Jackson ImmunoResearch) or Alexa Fluor 594- (Invitrogen, Grand Island, NY, USA) conjugated secondary antibodies against the corresponding species, for 2 h at room temperature. To confirm the apoptotic phenotype, we used a rabbit polyclonal active caspase-3 antibody (R&D Systems, Lille, France; 1:100). No staining was observed in control sections, where primary antibody was replaced by blocking buffer. Immunofluorescence analysis was performed using an epifluorescence microscope (Leica DM R HC model, Wetzlar, Germany). The data sets were acquired by Adobe Photoshop CS5 software (Adobe Systems, Inc., San Jose, USA) and images were subsequently processed with Image J open source software (version 1.46r).

Once determined, the apoptotic index in the adrenal cortex and medulla and the score of necrosis were analyzed through statistical analysis, as described below.

3.4. Statistical Analysis

Statistical analyses were carried out with the Statistica software, version 8.0 (Statsoft Ibérica, Lisboa, Portugal). To perform the statistical analysis, a mixed linear model was applied to the data. Data was expressed as mean and standard error. For each variable, a two way ANOVA was used where variance components were obtained through the Variance Estimation and Precision (VEPAC) Statistica module. Variance components in the model were estimated by Restricted Maximum Likelihood (REML) estimation. Least Squares means and standard errors for Least square means were computed from the solution to the mixed model equations. Correlation analysis between variables was performed with the Spearman rank correlation, considering the complete set of four time points. Statistical significance was set at a $p < 0.05$.

4. Results

In G0 and G1 it was possible to obtain data for analysis from all animals. However in 3 animals of G2 and 2 animals of G3, the data available for several variables and/or time points was insufficient to perform statistical analysis and consequently these animals were excluded

4.1. Hormonal variables

The statistical analysis of hormonal variables is shown in Figure 13. The statistical descriptive analysis of hormonal variables in described in Table 2. ACTH levels were always higher in G3 than in G2, except at T3 where the opposite occurred. ACTH levels were also higher in G3 than in G1 in T0, T1 and T2 and then G0 at all time points. ACTH was higher in G2 than in G1 at T1 and T2, but at T0 and T3 the opposite situation occurred. G0 had the lowest levels of ACTH when compared with the other three groups, in all time points. None of these differences reached statistically significance except the ones between G2 and G3 with G1 at T1 (G2 with G1, p =0.001; G3 with G1, p =0.000) and G2 and G3 with G0, also at T1 (G2 with G0, p =0.001; G3 with G0, p =0.000). In both HS groups, ACTH reached its highest level at T1 (after HS). From here, ACTH concentrations evolved differently between G2 and G3. In G3, the values decreased progressively until the end of the experiment. In G2, the values decreased from T1 to T2 and then increased again from T2 to T3. In G1 ACTH decreased from T0 to T1, remained stable from T1 to T2 and then increased again at T3. In G0 the values of ACTH decreased continuously from T0 to T3. The interaction between ACTH and time was found to be statistical significant (p=0.006).

Etomidate-treated rats had always levels of CS which were lower than in G2 although significant differences were only found at T1 (p =0.000) and T2 (p =0.031). They were also lower in G3 than in G1 at T0, T2 and T3 although the differences were only statistically significant at T0 (p =0.003) and T3 (p =0.001). When compared with G0, CS levels were higher in G3 at T1 and T3 and lower in T0 and T2. However none of these differences was statistically significant.

CS levels were higher in G2 than in G1 at T1 and T2, although only at T1 this was statistically significant (p =0.000). In contrast they were lower than G1 at T0 and T3, although only at T3 this was statistically significantly (p = 0.046). The levels of CS were always higher in G2 than in G0 at all time points, but none of the differences was statistically significant.

Finally CS was always higher in G1 than in G0, although the only difference statistically significant was at T3 (p=0.002).

CS highest levels were observed in G2 at T1. The lowest levels were observed in G3 at T2 and G0 at T3, which displayed similar levels. In G3, the levels of CS were lower than 10000 pg/ml at T0, T1 and T2. The interaction between CS and time was found to be statistical significant (p=0.015).

Figure 13: Variation of hormonal variables from T0 to T3 in G0, G1, G2 and G3

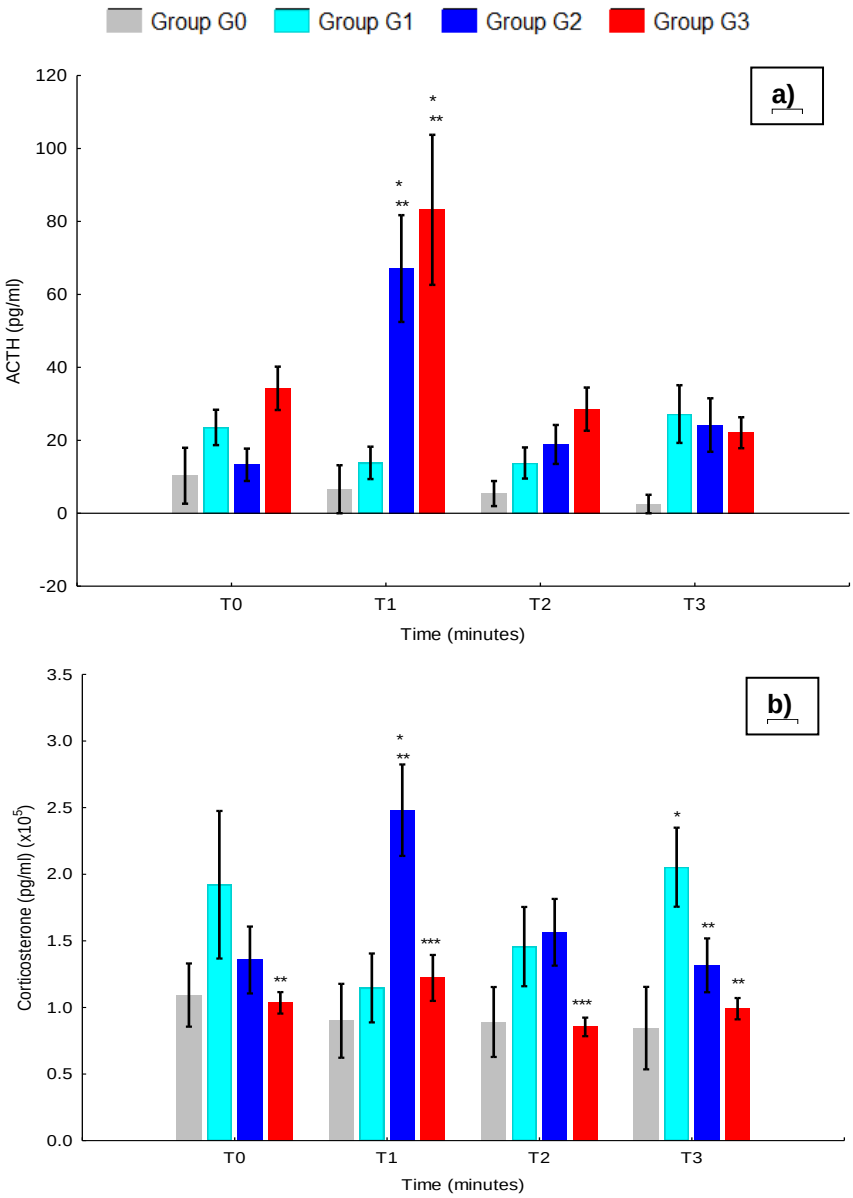

Figure 13: **a)** ACTH; **b)** CS. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. Data expressed as mean ± SE. * indicates statistically significant differences between G1, G2 or G3 and G0; ** indicates statistically significant differences between G2 or G3 and G1; *** indicates statistically significant differences between G3 and G2. Statistical significance was established at p-value < 0.05. ACTH: adrenocorticotropic hormone; CS: corticosterone.

4.2. Immunological variables

Immunological variables are displayed in Figure 14. The statistical descriptive analysis of immunological variables is described in Table 3. In G0, G1 and G2, TNF-α was always below the lower limit of detection. In contrast, etomidate-treated rats had detectable levels of TNF-α at all time points. However the differences between G3 with G0, G1 and G2 were only statistically significant at T2 (G3 with G0, p =0.017; G3 with G1, p =0.008; G3 with G2, p =0.012) and T3 (G3 with G0, p =0.040; G3 with G1, p =0.022; G3 with G2, p =0.030). The differences between G0, G1 and G2 were not statistically significant. In G3, the cytokine increased progressively until T2 and then decreased slightly at T3.

IL6 levels were always higher in G3 than in G1, G2 and G0. However these differences were only statistically significant at T3 (G3 with G0, p =0.004; G3 with G1, p =0.000; G3 with G2, p =0.002). IL6 levels were always higher in G2 than in G0 and G1 and in G0 than in G1, at all time points, although none of these differences was statistically significant. In G3, IL6 levels increased progressively from T0 to T3. By contrary, in G0, G1 and G2 they remained stable along the experiment.

IL10 levels were above the limit of detection in G2 and G3 only. They were also higher in G3 than in G2 at all time points, although both HS groups only differ in a statistically significant way at T3 (p = 0.004). A statistically significant difference was found between the levels of IL10 of G3 with G1 at T2 (p=0.001) and T3 (p=0.002) and the levels of G3 and G0 at T1 (p=0.051), T2 (p=0.003) and T3 (p=0.005). No difference between G2, G1 and G0 at any time point was found to be statistically significant. Interestingly the evolution of IL10 levels along the experiment was similar in both HS groups, with IL10 increasing progressively from T0 toT2. In T2 it reached its maximum peak and from here they decreased in T3.

Figure 14: Variation of immunological variables from T0 to T3 in G0, G1, G2 and G3.

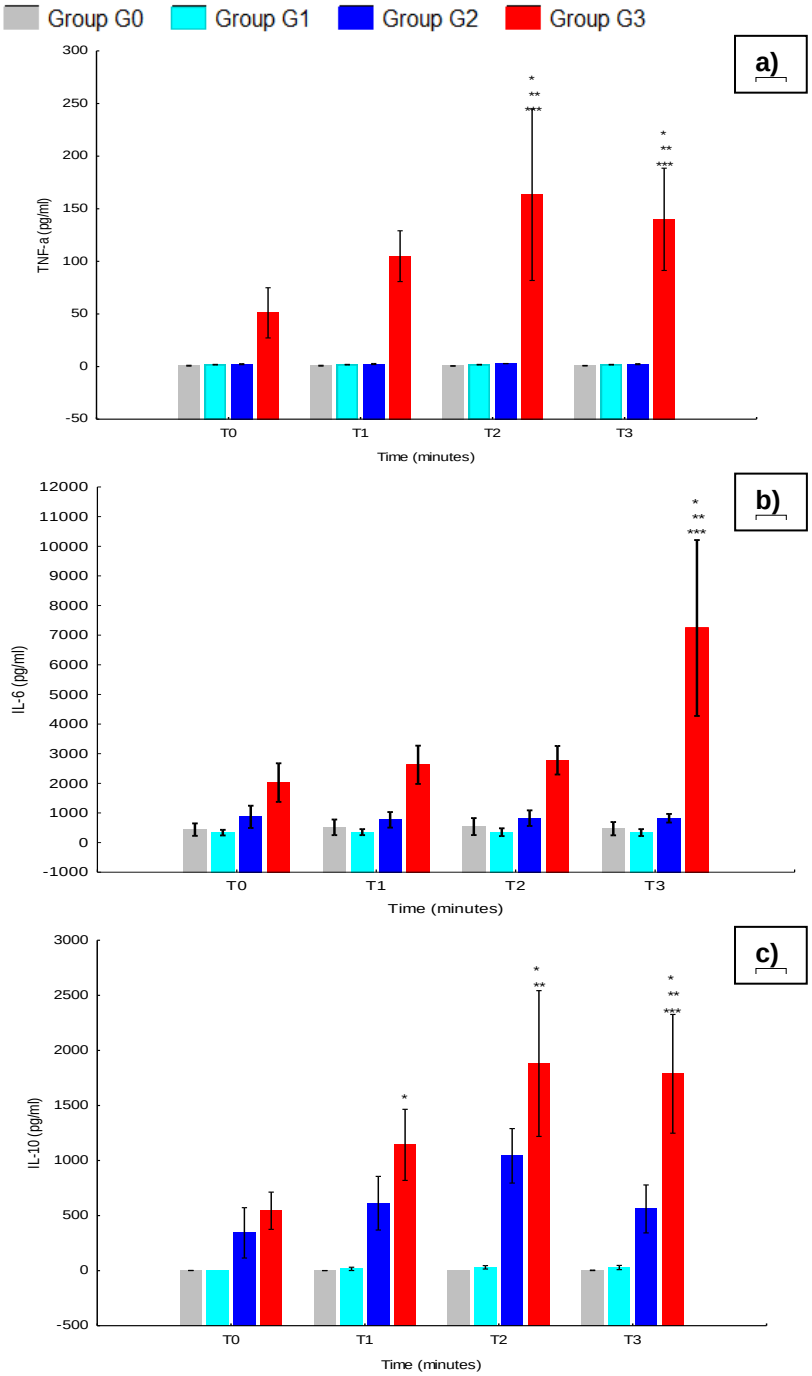

Figure 14**: a)** TNF-α; **b)** IL6; **c)** IL10. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. Data expressed as mean ± SE. * indicates statistically significant differences between G1, G2 or G3 and G0; ** indicates statistically significant differences between G2 or G3 and G1; *** indicates statistically significant differences between G3 and G2. Statistical significance was established at p- value < 0.05. TNF-α: Tumor Necrosis Factor-α; IL6: Interleukin 6; IL10: Interleukin 10.

4.3. Metabolic variables

Metabolic variables are displayed in Figure 15 and 16. The statistical descriptive analysis of metabolic variables is described in Table 4.

Both HS groups had lower values of pH than G1 and G0 at all time points. G3 had lower pH than G2 in all time points except T0, although none of the differences between both HS groups was found to be statistically significant. The difference between G3 and G1 was statistically significant at T1 (p=0.001), T2 (p=0.000) and T3 (p=0.000). G3 also differed significantly from G0 at the same time points, with the correspondent level of significance being: T1 (p=0.004), T2 (p=0.000) and T3 (p=0.004). The differences between G2 and G1 were also found to be statistically significant at T1 (p=0.003), T2 (p=0.000) and T3 (p=0.000). G2 also differed significantly from G0 at T1 (p=0.009) and T2 (p=0.029) .The pH was always higher in G1 than in the other groups, except at T0, where its levels were highest in G0.The differences between G1 and G0 were not statistically significant except at T3 (p=0.003) In both HS groups, pH decreased as the experiment progressed, with its lowest level being achieved at T3. In G1, the opposite occurred, with pH increasing progressively from T0 to T3, where it reached its highest level. In G0, after a first increase from T0 to T1, pH levels decreased continuously until T3.

Lactate levels were higher in G3 than in G2 at all time points, although none of the differences was found to be statistically significant. The levels of lactate were also higher in G3 than in G1 at all time points except T0. However the differences were only statistically significant at T1 (p =0.000) and T3 (p = 0.013). The levels of lactate in G3 were also higher than in G0, although only at T1 this difference was statistically significant (p=0.000). G2 had also higher levels of lactate then G1 and G0 except at T0. However, the only statistically significant difference was found between G2 and G0 at T1 (p=0.002). Lactate levels were higher in G1 than in G0 at T0 and T1. At T2 and T3 the situation reversed. However G1 and G0 did not differ significantly in any of the time points. The evolution of lactate levels was similar in both groups with HS, reaching its highest level after hemorrhage, decreasing with resuscitation and having a last increase from T2 to T3. In G1 lactate had a modest increase until T1, and then decreased to its lowest level at T3. In G0, lactate increased continuously until T2, when it reached its peak. It then decreased from T2 to T3.

BE values were always lower in G3 than in G2 and G1 at all time points. G3 differed significantly from G2 at T1 (p=0.013), T2 (p=0.020) and T3 (p=0.008) and from G1 also at T1 (p=0.000), T2 (p=0.000) and T3 (p=0.000). When compared with G0, the levels of BE were lower at T1, T2 and T3, differences that reached statistical significance [T1 (p=0.000), T2 (p=0.001) and T3 (p=0.00)]. However at T0, the levels of BE were very similar between G3 and G0, without a significant difference. The levels of BE in G2 were always lower than G1 at all time points, although this difference was only statistically significant at T1 (p=0.001), T2

(p=0.001) and T3 (p=0.000). When compared with G0, G2 had always lower levels of BE, except at T0, when the opposite occurred. None of the differences between G2 and G0 reached statistical significance. The levels of BE in G1 were the highest from all groups and at all time points. They differed from the levels of G0 in a statistically significant way at T0 (p=0.046), T2 (p=0.019) and T3 (p=0.000). In both HS groups, BE had a marked decrease from T0 to T1, then it improved slightly with resuscitation at T2 and suffered a last decrease from T2 to T3. In G0, BE decreased progressively from T0 until the end of the experiment. In G1, BE was more stable and had minimal variations between time points. An interaction between time and BE was also found to possess statistical significant (p=0.016).

The results of HCO_3 paralleled those of BE differing only in small aspects. At T0, the levels of HCO_3 were very similar between G1 and G2, although G1 had the highest levels. This was followed by G3 and finally by G0, which had the lowest levels. However, none of these differences between groups was found to be statistically significant. From T1 to T3, HCO_3 levels were always lower in HS groups than G1 at all time points. The levels of HCO_3 of G3 were also lower than those of G0 from T1 to T3. If one compares both HS groups, it becomes clear that HCO_3 was lowest in G3 than in G2, at all time points, although these differences were only statistically significant at T1 (p =0.013), T2 (p =0.020) and T3 (p =0.006). G3 also differed significantly from G1 at T1 (p =0.000), T2 (p =0.000) and T3 (p =0.000) and from G0 at the same time points [T1 (p =0.000), T2 (p =0.004) and T3 (p =0.008)]. The differences between G2 and G1 were statistically significant at T1 (p =0.025), T2 (p = 0.028) and T3 (p = 0.006). Although the levels of HCO_3 of G2 were lower than G0 at T1, T2 and T3, these differences were not statistically significant. G1 had the highest levels of HCO_3 of all groups. However when compared with G0, only at T3 the difference was statistically significant (p=0.003). In both HS groups, HCO_3 suffered a marked decrease from T0 to T1 (with HS), which was followed by a brief recovery from T1 to T2 (with resuscitation) and a subsequent decrease from T2 to T3. In G1, HCO_3 levels slightly decreased from T0 to T1 and then remained stable, with just some mild variations, until the end of the experiment. In G0, the levels of HCO_3 decreased progressively from T0 to T3.

The levels of PO_2 in G3 were always lower than in G2 and G1 at all time points. G3 differ significantly from G2 at T2 (p =0.012) and T3 (p =0.007) and from G1 at T0 (p =0.008), T2 (p =0.000) and T3 (p =0.001). In comparison with G0, the levels of PO_2 were always lower in G3, except at T1, when the opposite occurred. Nevertheless none of the differences found between G3 and G0 were statistically significant. When comparing the levels of PO_2 between G2 with G1 and G0, it was found that G1 had the highest levels, followed by G2 and only after by G0. However none of the differences between G2 with G1 or G0 was found to be statistically significant. The levels of PO_2 in G1 where significantly higher than those in G0 at T0 (p =0.035), T1 (p =0.013) and T2 (p =0.013). In G3, the levels of PO2 begin to decrease

slightly from T0 to T1. However from T1 to T2 (resuscitation) the decrease became more pronounced, with PO_2 reaching its lowest level at T3. In G2, the behavior of PO_2 was different. A marked decrease was observed from T0 to T1 (with HS). This was followed by a tendency of PO_2 levels to stabilize (they even improved slightly from T1 to T2). In G1, PO_2 levels decreased continuously from T0 to T3. Finally in G0, after a progressive decrease from T0 to T2, PO_2 levels improved slightly from T2 to T3. The results of PO_2/FiO_2 ratio were similar to those described for PO_2. In etomidate-treated rats, the PO_2/FiO_2 ratio decreased progressively from T0 to T3, reaching values below 300 at T2 and T3.

Figure 15: Variation of metabolic variables (pH, lactate, BE and HCO_3) from T0 to T3 in G0, G1, G2 and G3

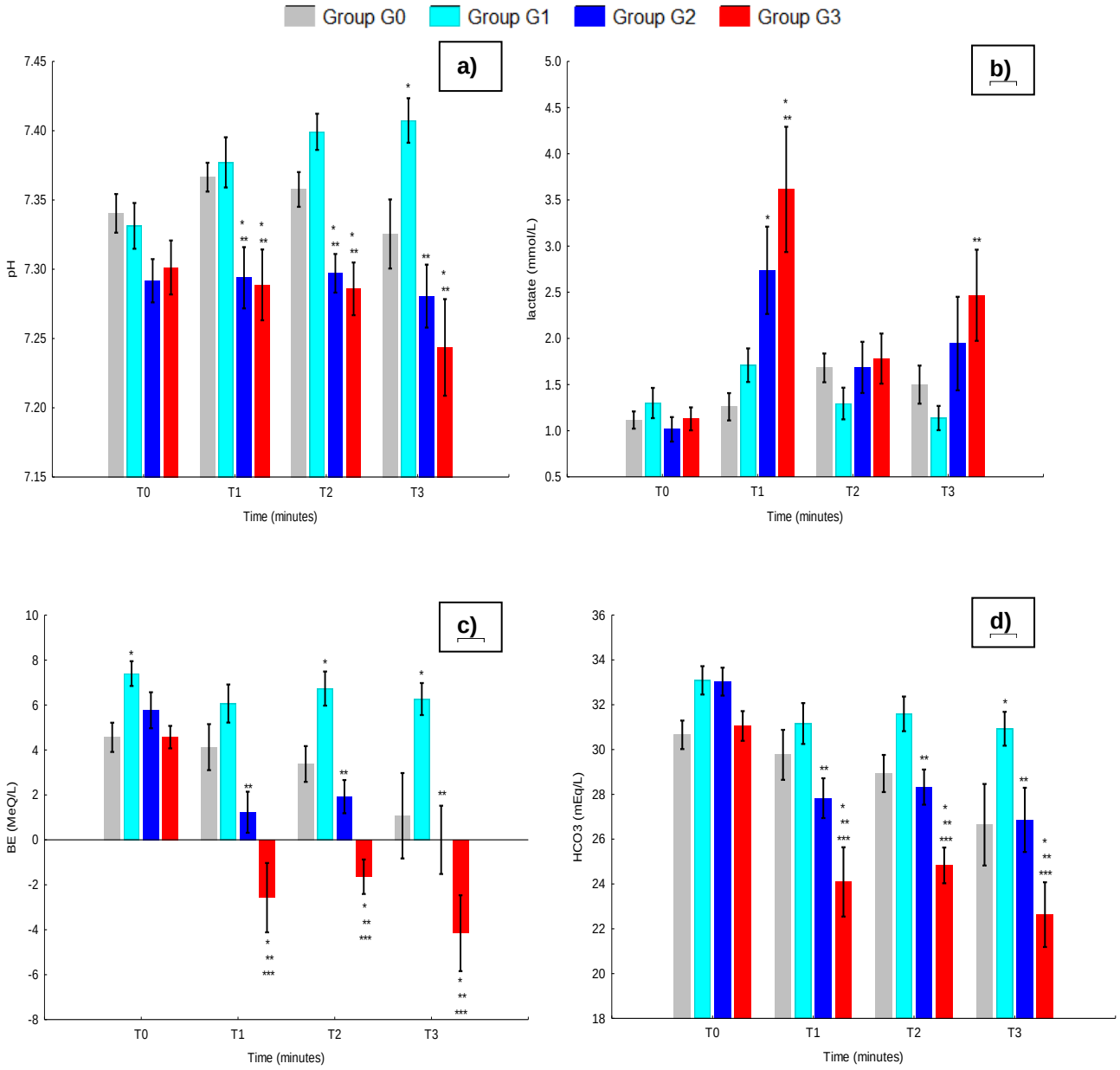

Figure 15: **a)** pH; **b)** lactate; **c)** BE; **d)** HCO_3. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. Data expressed as mean ± SE. * indicates statistically significant differences between G1, G2 or G3 and G0; ** indicates statistically significant differences between G2 or G3 and G1; *** indicates statistically significant differences between G3 and G2. Statistical significance was established at p- value < 0.05. BE: base excess.

Figure 16: Variation of metabolic variables (PO_2, PO_2/FiO_2) from T0 to T3 in G0, G1, G2 and G3.

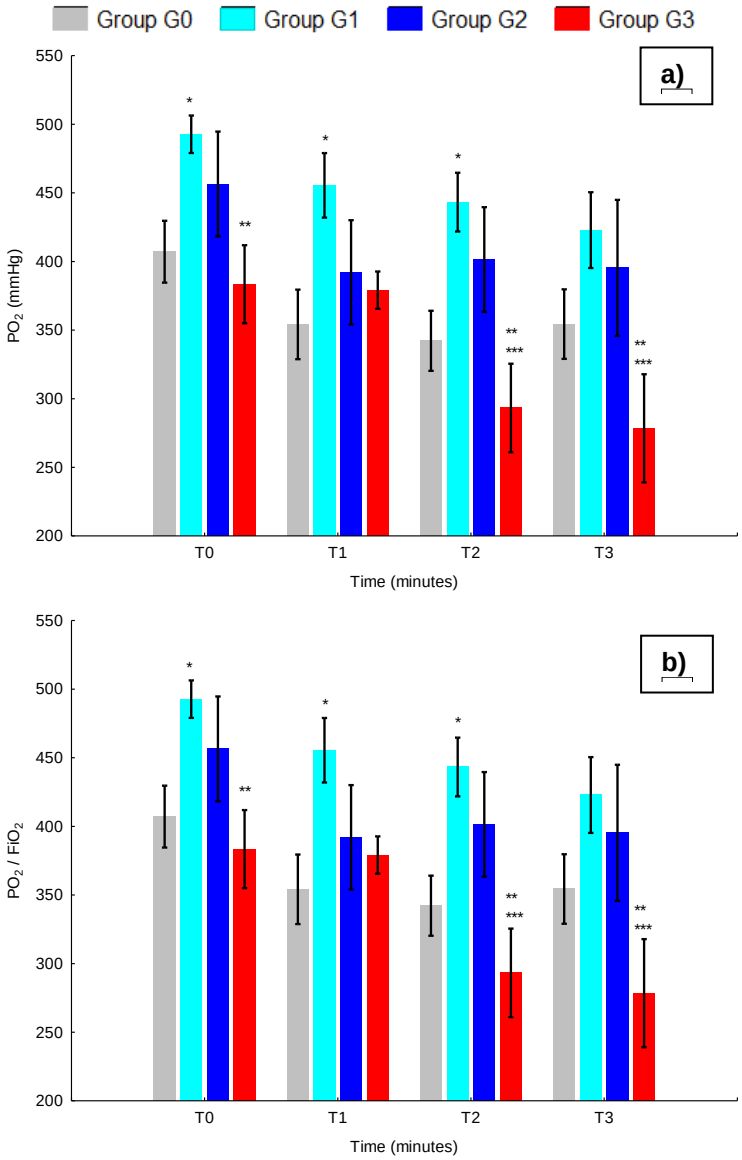

Figure 16: Variation of metabolic variables (PO_2 and PO_2/ FiO_2) from T0 to T3 in G0, G1, G2 and G3. **a)** PO_2 **b)** PO_2/ FiO_2. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. Data expressed as mean ± SE. * indicates statistically significant differences between G1, G2 or G3 and G0; ** indicates statistically significant differences between G2 or G3 and G1; *** indicates statistically significant differences between G3 and G2. Statistical significance was established at p- value < 0.05.

4.4. Hemodynamic variables

The analysis of hemodynamic variables is shown in Figure 17 and the statistical descriptive analysis in Table 5. HR was always lower in G3 than in G2, a difference which was statistically significant at T2 (p =0.037) and T3 (p =0.003). It was also lower in G3 than in G1 at T1, T2 and T3 but the differences were not statistically significant. The comparison between G3 and G0 also revealed a lower HR in G3 at all time points, although none of the differences was found to be statistically significant. G2 had higher levels of HR than G0 at all time points and then G1 at T0, T1 and T3 but no differences reach statistical significance. G1 had a lower HR than G0 at T0 and T1, but the opposite occurred at T2 and T3 (although in this the values of HR were very similar). Again, no statistically significant difference was found between G1 and G0. In G3, HR was stable between T0 and T1, and then decreased continuously until the end of the experiment. In G2, HR began to decrease at T0 to T1, a tendency which progressed until the end of the experiment. The decrease was not as marked as the one observed in G3. In G1, HR increased from T0 to T2 and then decreased slightly from this to T3. In G0, HR levels were stable from T0 to T2 and then decreased at T3. Regarding MAP, no statistically significant differences were found between groups, except between G2 and G3 at T0. At this time point, MAP was significantly lower in etomidate-treated animals (p=0.014). When comparing both HS groups, at T0 and T1, MAP was higher in G2 than in G3, but the opposite occurred at T2 and T3. At T2, the MAP of etomidate-treated animals was higher than the MAP of any of the other groups and at T3 it was only exceeded by G0. G2 animals had the highest MAP values at T0 from all groups. However, as the experience progressed, MAP decreased continuously from T0 to T3 and at the last time point, this group was actually the one with the lowest MAP levels. In G0, MAP decreased continuously from T0 to T2, and then slightly increased at T3. In contrast, in G1, MAP decreased continuously from T0 to T3. In G3, it decreased from T0 to T1 after HS, increased with resuscitation (from T1 to T2) and suffered a new decrease from T2 to T3.

Figure 17: Variation of hemodynamic variables (HR, MAP) from T0 to T3 in G0, G1, G2 and G3.

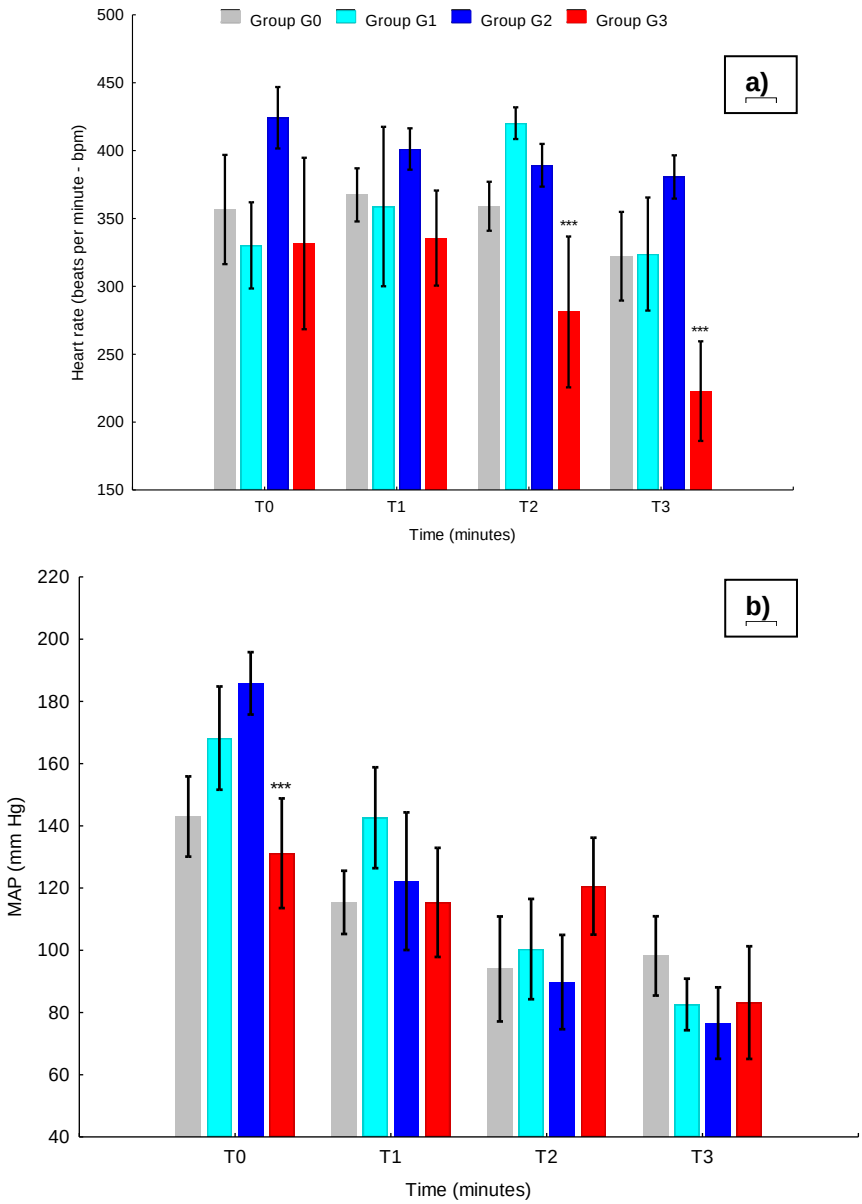

Figure 17: Variation of hemodynamic variables from T0 to T3 in G0, G1, G2 and G3. **a)** HR; **b)** MAP. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. Data expressed as mean ± SE. * indicates statistically significant differences between G1, G2 or G3 and G0; ** indicates statistically significant differences between G2 or G3 and G1; *** indicates statistically significant differences between G3 and G2. Statistical significance was established at p- value < 0.05.HR: heart rate; MAP: mean arterial pressure.

4.5. Molecular biology variables

Molecular biology variables are shown in Figure 18 and its statistical descriptive analysis is described in Table 6. We couldn´t obtain mRNA from all rats at several time points. In addition there was a large variability in the results.

The levels of *TNFα* mRNA were higher in G3 than in the other groups at all time points. Statistically significant differences were found between G3 and G1 at T0 ($p = 0.020$) and between G3 and G2 ($p =0.033$), G3 and G1 ($p =0.039$) and G3 and G0 ($p=0.010$) at T1. Although *TNFα* mRNA was numerically higher in G2 than in G1 no statistically significant differences were found between these two groups. The highest value of *TNFα* mRNA expression was observed in G3 at T1, after hemorrhage. This was followed by a progressive decrease of its levels until the end of the experiment. In the other three groups, the expression *of TNFα* mRNA was highly variable to preclude any analysis.

Regarding *IL6* mRNA, few statistically significant differences were found and they were limited to T0. These include the difference between *IL6* mRNA of G3 and G1 ($p=0.049$), G2 with G0 ($p=0.026$) and between G1 with G0 ($p=0.020$). When comparing both HS groups, *IL6* mRNA levels were always higher in G3 than in G2 at all time points. In G3, *IL6* mRNA increased from T0 to T1, slightly decreased from T1 to T2 and then remained stable, at high levels until the end of the experiment. In G2, the pattern of expression was similar, except that the rise of *IL6* mRNA from T0 to T1 was much more marked. When comparing G1 with G0, the latter had always higher levels of *IL6* mRNA. In G1, *IL6* mRNA increased markedly from T0 to T1, stabilized from T1 to T2 and decreased slightly at T3. G0 had the highest levels of *IL6* mRNA in all time points and they remained stable along the experiment, only presenting a slight decrease at T3. G3 was the next group with the highest *IL6* mRNA at T0, T1 and T3. G2 had the lowest levels of *IL6* mRNA of all groups at all time points except T0.

Regarding *IL10* mRNA only one difference between groups was found to be statistically significant. This was between G2 and G0 at T1 ($p=0.045$). When comparing the levels of *IL10* mRNA between the two HS groups, it was found that it was always higher in G3 than in G2, except at T0, where the opposite occurred. In G3, *IL10* mRNA increased from T0 to T1 and then slightly decreased until the end of the experiment. In contrast, in G2, it had a marked decrease from T0 to T1, followed by a pronounced increase at T2 (almost reaching the levels of T0) with resuscitation and finally ended with another marked decrease from T2 to T3. Both G0 and G1 had high levels of *IL10* mRNA at T0, T1 and T2 and both showed a marked decrease of this variable from T2 to T3. The levels of *IL10* mRNA were higher in G1 than in G0 at T0 and T2, with the opposite occurring at the remaining time-points.

Figure 18: Variation of molecular biology variables from T0 to T3 in G1, G2 and G3.

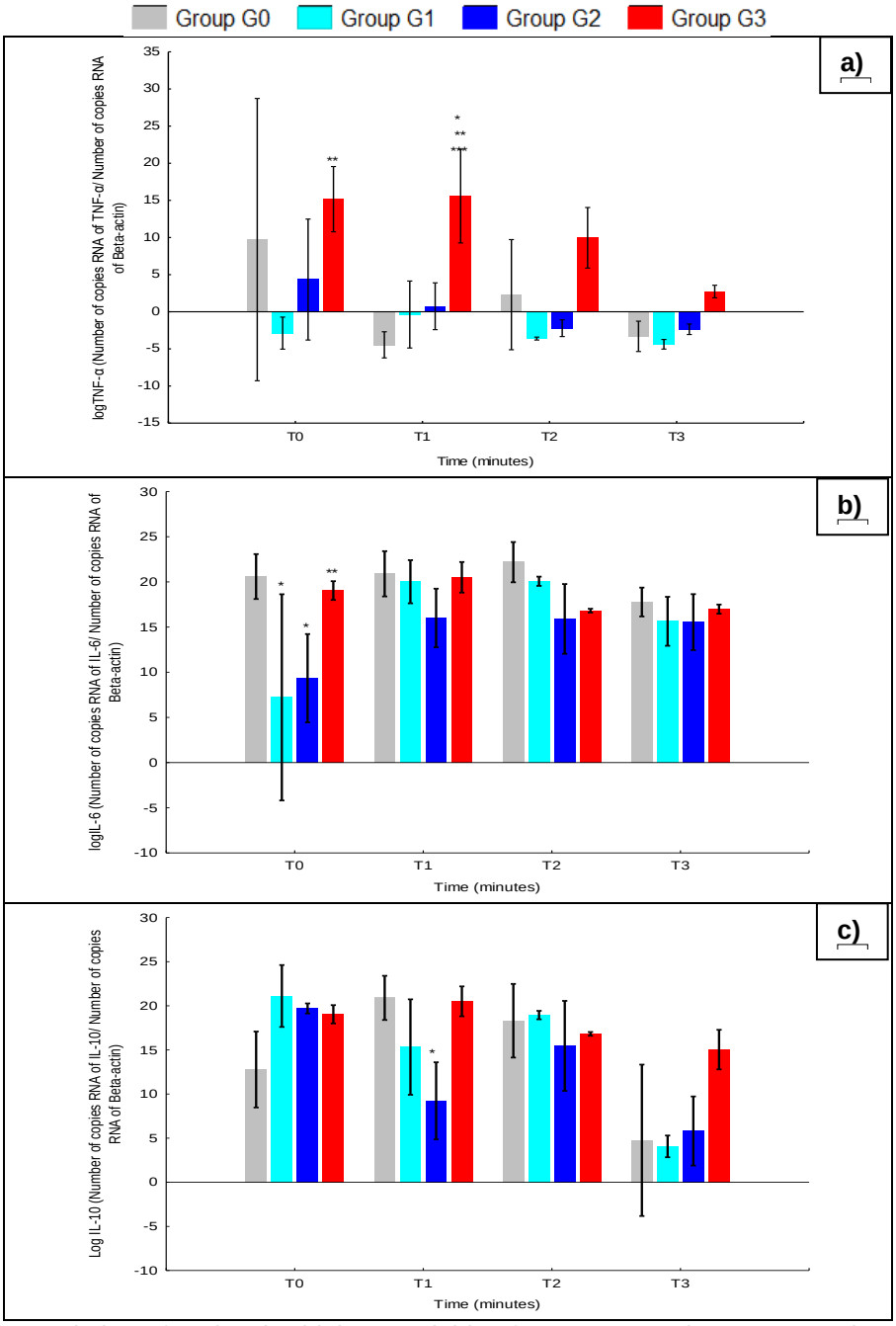

Figure 18: Variation of molecular biology variables from T0 to T3 in G1, G2 and G3. **a)** logTNF-α;**b)** logIL6;**c)** logIL10. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. Data expressed as mean ± SE. * indicates statistically significant differences between G1, G2 or G3 and G0; ** indicates statistically significant differences between G2 or G3 and G1; *** indicates statistically significant differences between G3 and G2. Statistical significance was established at p- value < 0.05.

4.6. Δvolume

Δvolume values for G2 and G3 groups are shown in Figure 19. Although Δvolume was higher in G3 than in G2, the difference was not statistically significant.

Figure 19: Δvolume in G2 and G3.

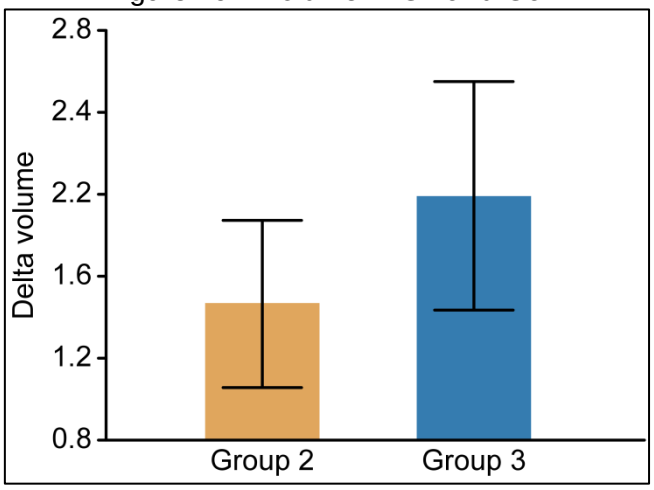

Figure 19: Δvolume in G2 and G3. G2: animals submitted to general anesthesia, mechanical ventilation, buprenorphine administration and shock; G3: similar to G2 but also with etomidate administration. Statistical significance was established at a p-value < 0.05.

4.7. Histopathological variables

4.7.1. Apoptosis

The statistical descriptive analysis of cortical and medullary apoptotic index for the different groups is described in Table 7. Figure 20 shows the statistical analysis for apoptotic index and of the different groups. Figure 21 displays images of TUNEL-positive nuclei in the adrenal cortex and medulla and cortical cells staining positive for active caspase-3 immunohistochemistry for groups G0 and G1. Figure 22 displays the same features, but in this case for groups G2 and G3. Figure 23 shows TUNEL-positive nuclei in an area of transition between the cortex and medulla in an animal belonging to G2. Figure 24 display TUNEL positive cells at high resolution (1000x), to demonstrate that adrenocortical cells are the major cell types affected by apoptosis.

In all groups the apoptotic index was higher in the cortex than in the medulla. The highest cortical apoptotic index was observed in G2. The second highest apoptotic index was observed in G3, followed by G1 and G0. G0 differed in a statistically significant way from G1 (p=0.039), G2 (p=0.000) and G3 (p=0.000). G1 also differed significantly from G2 (p=0.008). The difference between G2 and G3 was not statistically significant.

The medullary apoptotic index was highest in G2, a difference which was statistically significant from G0 (p=0.000), G1 (p=0.000) and G3 (p=0.000). G3 had the second highest medullary apoptotic index, followed by G0 and G1. Nevertheless, none of the differences between G0, G1 and G3 groups were statistically significantly.

In the cortex, TUNEL-positive nuclei were more frequently found in inner cortical zones, near the medulla, in an area corresponding to the zone reticularis in Humans (Figure 9 and 10). Some authors suggested that rats do not possess zone reticularis (Pihlajoki, Dörner, Cochran, Heikinheimo & Wilson, 2015). However this is not the most commonly accepted view (Vinson, 2016) and consequently we opted to maintain the term “zone reticularis” to designate the most inner portion of the rat´s adrenocortical cortex.TUNEL-positive nuclei were also observed in *zone fasciculata*, although less frequently than in *zone reticularis*. They were infrequently observed in *z*one *glomerulosa.*

Figure 20: Variation of the apoptotic index G0, G1, G2 and G3

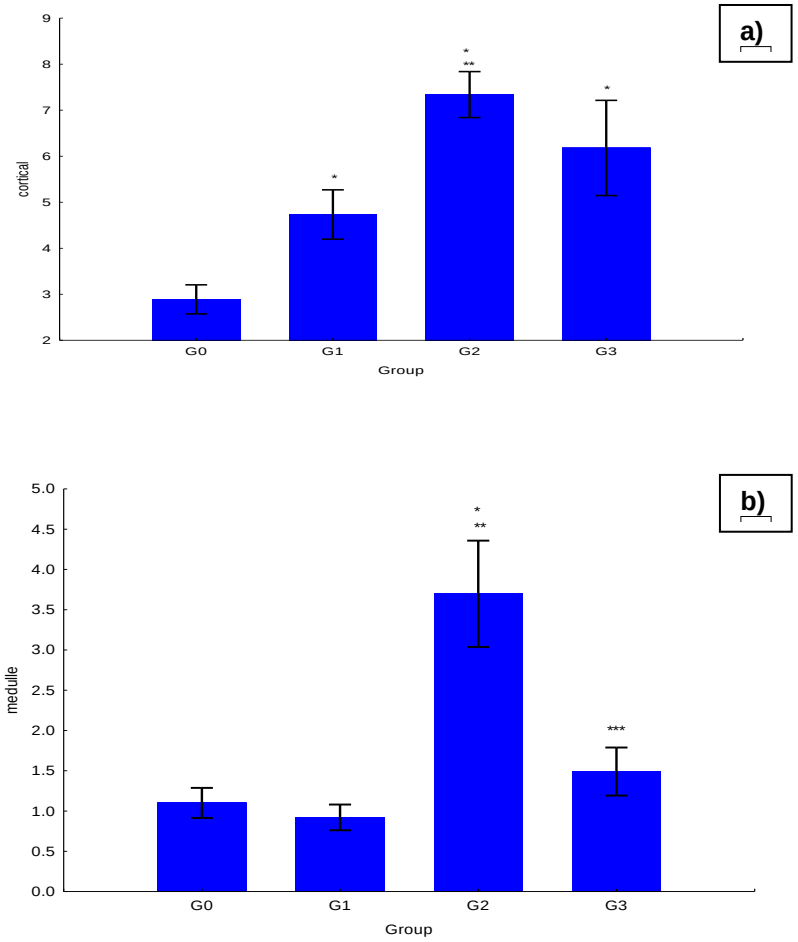

Figure 20: Apoptosis score in G0,G1, G2 and G3. **a)** apoptosis score in the adrenal cortex; **b)** apoptosis score in the adrenal medulla. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. Data expressed as mean ± SE. * indicates statistically significant differences between G1, G2 or G3 and G0; ** indicates statistically significant differences between G2 or G3 and G1; *** indicates statistically significant differences between G3 and G2. Statistical significance was established at p- value < 0.05.

Figure 21: Pictures of TUNEL positive nuclei in the adrenal cortex and medulla and caspase-3 immunofluorescence analysis in G0 and G1.

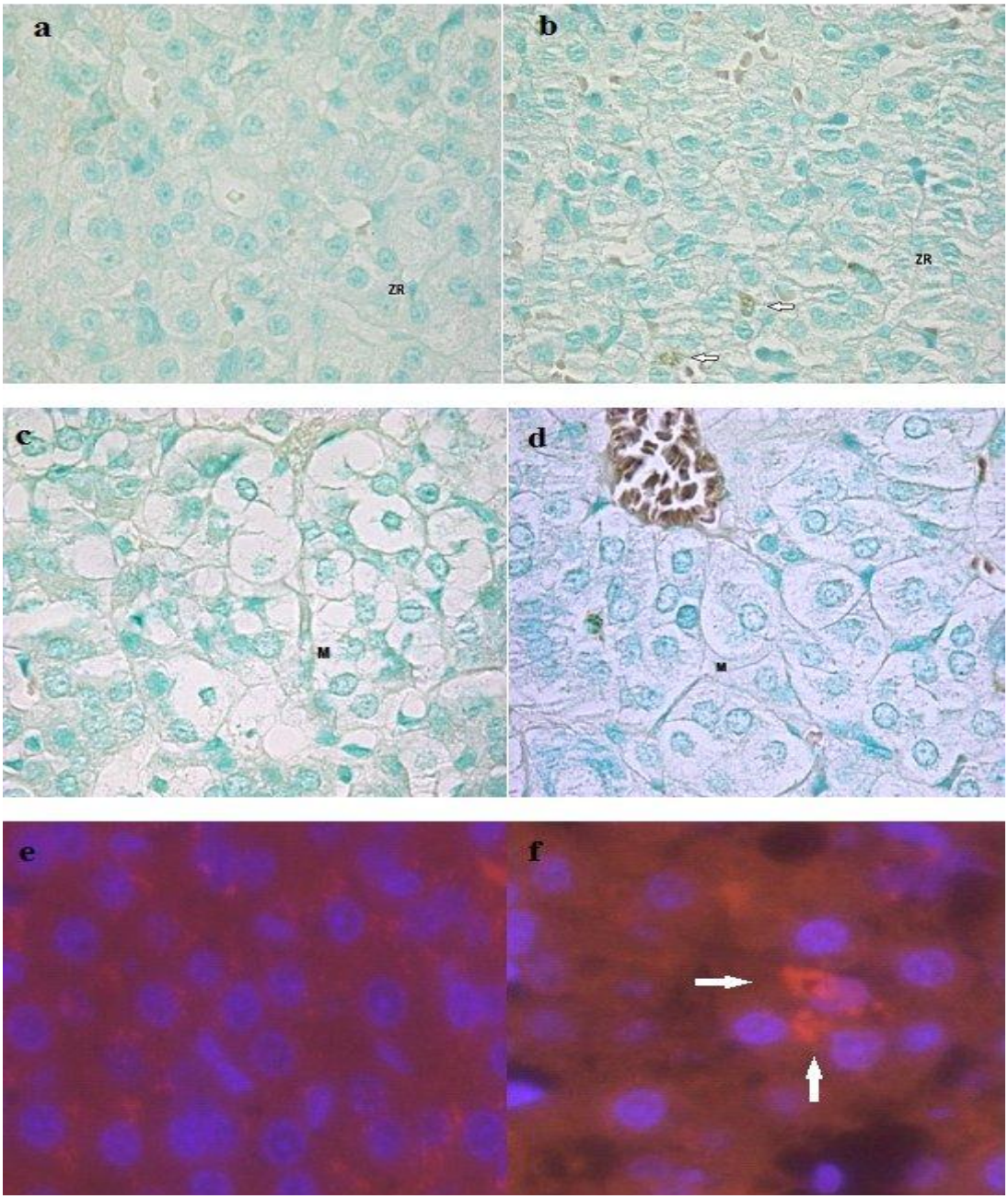

Figure 21: First row corresponds to sections of adrenal cortex; second row corresponds to sections of adrenal medulla; third row corresponds to pictures of caspase-3 immunofluorescence analysis in the adrenal cortex. The column of the left corresponds to G0 and the column of the right to G1. Pictures were taken at 400x magnification. **a)** TUNEL in adrenal cortex of G0; **b)** TUNEL in adrenal cortex of G1; **c)**TUNEL in adrenal medulla of G0 ; **d)** TUNEL in adrenal medulla of G1; **e)** caspase-3 immunofluroescent analysis in G0; **f)** caspase-3 immunofluroescent analysis in G1. Small white arrows in **b** indicate adrenal cortical cells from G1which stained TUNEL positive. Small white arrows in **f** indicate caspase 3 positive areas in adrenal cortical cells from G1, which appear with bright red. **ZR**-*zona reticularis*; **M**-medulla.

Figure 22: Pictures of TUNEL positive nuclei in the adrenal cortex and medulla and caspase-3 immunofluorescence analysis in G2 and G3.

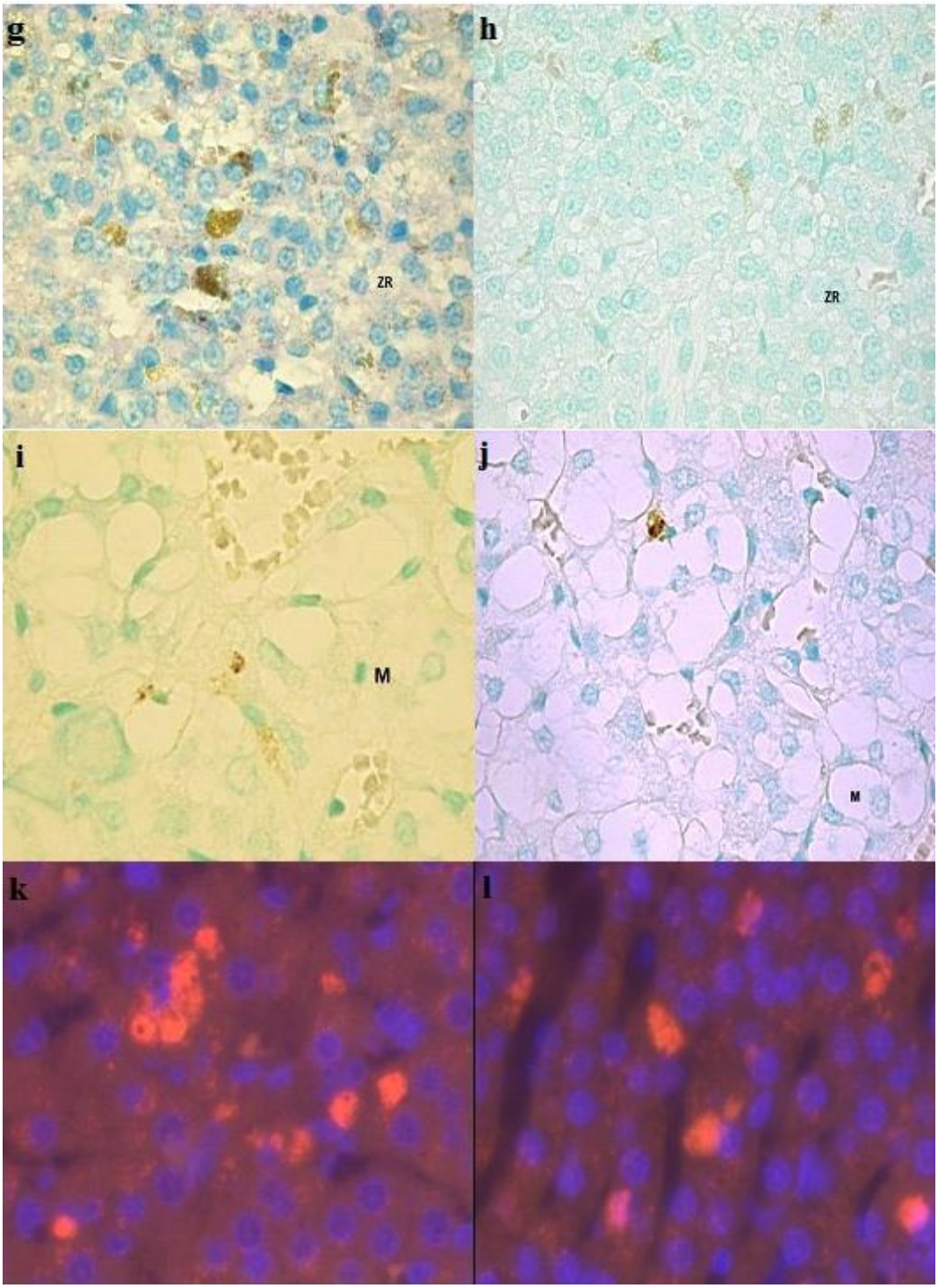

Figure 22: First row corresponds to sections of adrenal cortex; second row corresponds to sections of adrenal medulla; third row corresponds to pictures of caspase-3 immunofluorescence analysis in the adrenal cortex. The column of the left corresponds to G2 and the column of the right to G3. Pictures were taken at 400x magnification. **g)** TUNEL in adrenal cortex of G2; **h)** TUNEL in adrenal cortex of G3; **i)**TUNEL in adrenal medulla of G2 ; **j)** TUNEL in adrenal medulla of G3; **k)** caspase-3 immunofluroescent analysis in G2; **l)** caspase-3 immunofluroescent analysis in G3. **ZR**- *zona reticularis*; **M**-medulla.

Figure 23: TUNEL positive cells in both adrenal cortex (*zona reticularis*) and medulla from an animal of G2.

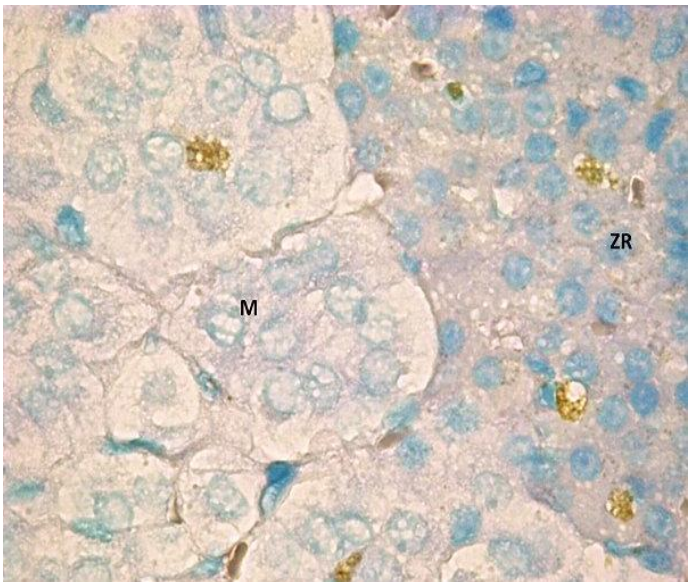

Figure 23: The picture shows that the adrenal cortex had a higher number of TUNEL positive cells than the medulla. Picture take at a 400x magnification ZR- *zona reticularis*; M-medulla.

Figure 24: TUNEL positive adrenal cells in high resolution

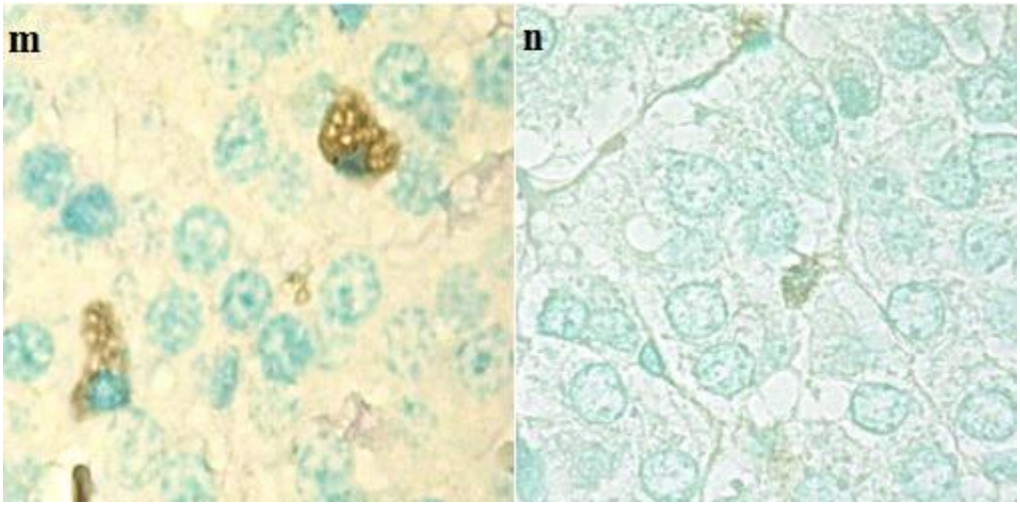

Figure 24: TUNEL positive adrenal cells in high resolution ; m) *zona reticularis* of an animal from G2; n) medulla from an animal of G3.

4.7.2. Necrosis assessment

The statistical analysis for the presence of necrosis in the different groups is displayed in figure 25 and Table 7. The only groups which displayed adrenal necrosis were G2 and G3, with highest score being found in G2, with a median of 2 (G3 animals had a mild degree of necrosis, with a score which was almost 0). The difference between the necrosis´s score in G2 was statistically significant with those found in G0 (p=0.000), with G1 (p=0.000) and with G3 (p=0.000). The score of necrosis also differed significantly between G3 and G0 (p=0.034).

Figure 25: Variation of the necrosis score in G0, G1, G2 and G3.

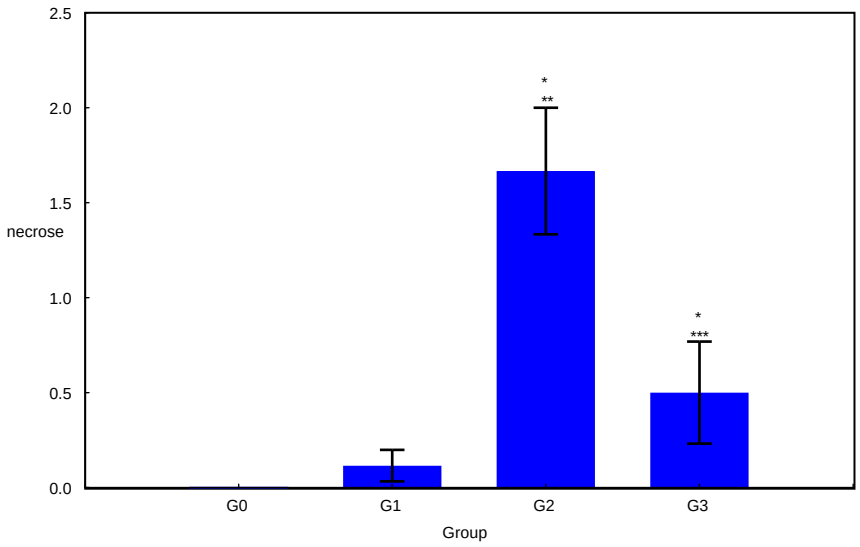

Figure 25: Necrosis score in G0, G1, G2 and G3. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. Data expressed as mean ± SE. * indicates statistically significant differences between G1, G2 or G3 and G0; ** indicates statistically significant differences between G2 or G3 and G1; *** indicates statistically significant differences between G3 and G2. Statistical significance was established at p- value < 0.05.

4.8. Correlation analysis

The correlation analysis is displayed in table 8, 9, 10, 11, 12, 13, 14 and 15. In these tables only statistically significant correlations are displayed. The complete set of values can be found in supplementary tables.

ACTH and CS were positively correlated with each other in both HS groups (p = 0.012, r = 0.506 for G2 and p = 0.001, r = 0.444 for G3). In G2, ACTH was positively correlated with TNFα (p = 0.048, r = 0.409), IL10 (p = 0.013, r = 0.503) and lactate (p = 0.000, r = 0.678) and negatively correlated with HCO_3 (p = 0.004, r = -0.566) and BE (p = 0.004, r =-0.572). TNFα was also positively correlated with IL10 (p = 0.009, r = 0.524) and negatively correlated with pH (p = 0.024, r = -0.463). IL10 was negatively correlated with HCO_3 (p = -0.418, r = 0.043) and BEecf (p = -0.440, r = 0.032) and positively correlated with lactate (p = 0.422, r =0.041).

In G3, besides CS, ACTH was also positively correlated with lactate (p = 0.031, r = 0.300). In this group, CS was also correlated with IL10 (p = 0.032, r = 0.298). TNFα was positively correlated with IL6 (p = 0.000, r = 0.558), and IL10 (p = 0.000, r = 0.706) and negatively correlated with pH (p = 0.001, r = -0.435), HCO_3 (p = 0.000, r =-0.464) and BE (p = 0.000, r = -0.548). IL6 was positively correlated with IL10 (p = 0.000, r = 0.575) and negatively correlated with pH (p = 0.016, r = -0.325), HCO_3 (p = 0.000, r =-0.466) and BE (p = 0.000, r = -0.502). IL10 was negatively correlated with pH (p = 0.000, r = -0.500), HCO_3 (p = 0.001, r = -0.444) and BE (p = 0.000, r = -0.548).

The apoptotic rate of the cortex and the medulla were correlated in a statistically significant positive way (r= 0.290; p=0.041). In addition it was found that the cortical apoptotic rate was positively correlated with the rate of necrosis (r= 0.426; p=0.002). The correlation between the medullary apoptotic rate and necrosis was not significant. None of the study variables was found to be correlated in a statistically significant way with the cortical and medullary apoptotic indexes (table 15). IL10 was found to have a positive correlation (r =0.6001; p = 0.002) and BE a negative correlation (r =0.4401; p = 0.009) with the adrenal score of necrosis.

5. Discussion

The results of the study confirmed some of our original hypotheses but not others. It was clearly demonstrated that HS was associated to development of adrenal gland apoptosis and necrosis. Confirming another of our hypotheses, etomidate administration was associated to development of CIRCI and this was associated to significant changes in hormonal, immunological, metabolic, hemodynamic and molecular biology variables. To the best of our knowledge, some of our results had been never reported before in the research setting. One example is the increased development of lung injury in animals treated with etomidate. Etomidate was also found to modulate the development of adrenal apoptosis. To our knowledge, for the first time, it was demonstrated that it modulates adrenal gland necrosis as well. However, contrarily to our hypothesis, we could not demonstrate that HS itself was clearly associated to development of CIRCI. We were also not able to associate the development of adrenal gland apoptosis to CIRCI. Finally, it was found that buprenorphine administration was associated to significant changes in several parameters, including the development of adrenal apoptosis. In the next sections, a discussion of our findings will follow. For convenience we opted to describe the major findings found in each group and if appropriate to mention the major differences between groups.

5.1. Analysis of G1

As described previously, G1 was created to evaluate the effects of buprenorphine in variables which would also be addressed in the HS groups, such as hormonal, immunological and molecular biology variables. Although buprenorphine is known for decades, the mechanisms behind its actions are incompletely understood, a fact which is probably related with buprenorphine´s complex pharmacology (Lutfy & Cowan, 2004). Initially described as a partial μ agonist, it was found that it is also a κ antagonist, a δ simultaneous agonist and antagonist, a NOP receptor agonist and a ε antagonist (Lutfy & Cowan, 2004; Huang et al.¸ 2001). In addition, it has been shown that the three major buprenorphine´s metabolites, norbuprenorphine, buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide are also metabolically active and have a biological activity which is distinct from the parenteral drug (Brown et al., 2011; Huang, Kehner, Cowan, Liu-Chen, 2001). The latter most probably results from different receptor affinity (Brown et al., 2011).When compared with G0, it was found that animals of G1 had significantly higher levels of CS, pH, BE, HCO_3, PO_2 and $PO2/FiO_2$ and a significantly higher degree of apoptosis in the adrenal cortical cell´s. To our knowledge these findings have never been reported before.

5.1.1. Effects of buprenorphine in hormonal variables

The higher levels of ACTH and CS of G1 when compared with G0 were unexpected because most studies support the concept that buprenorphine does not affect or actually decreases HPA axis activity in rats (Goldkuhl et al., 2010; Goldkuhl et al., 2008; Gomez-Flores et al., 2000). One study described the influence of buprenorphine administered by intra-cerebral route in the HPA´s activity of anesthetized rats (Gomez-Flores et al., 2000). It found that buprenorphine was associated to decreased ACTH and CS levels, an effect which was attributed to buprenorphine´s partial agonist activity in opioid receptors. Another study showed that the administration of the opioid to non-operated rats was not associated to changes in CS levels (Pechnick et al., 1985). In addition, in several experimental models where buprenorphine was used to provide peri-operative analgesia, a decrease in the levels of CS was found during the post-operative period. This was mainly attributed to the ability of buprenorphine to decrease post-operative pain (Goldkuhl et al., 2008; Goldkuhl et al., 2010; Franchi, Panerai & Sacerdote, 2007). In contrast, a more recent study shown for the first time that animals which received buprenorphine for analgesia after abdominal surgery had higher levels of CS in the post-operative period than animals which were treated with ketorolac or placebo (Wilson, Ripsch & White, 2016). The mechanisms behind this increase were not established, but the authors suggested that this could be related with the higher levels of post-operative tactile hyperalgesia which were found buprenorphine-treated animals. This last study illustrates that the effects of buprenorphine in the peri-operative stress response to surgery are complex and still remain incompletely understood.

In our case we hypothesize that buprenorphine was the cause of increased ACTH and CS levels in G1 animals based in several assumptions. First, buprenorphine was the only factor in which G0 and G1 differed. Secondly, factors which are known to increase the activity of the HPA axis were similar between both groups. These include the type of surgical technique (Schricker et al., 1996), the severity of injury, the amount of blood collection and the total operating time (Goldkuhl et al., 2008; Vahl et al., 2005; Schricker et al., 1999; Schricker et al., 1996; Clarke et al., 1970). Regarding blood collection in particular, it comprised less than 15% of body weight and it was followed by volume replacement with normal saline. Studies have shown that in these conditions blood collection is not associated to increased HPA activity (Wiersma & Kastelijn, 1985). Thirdly, in G1 the time period when increased ACTH and CS levels were found is compatible with what is known about buprenorphine´s pharmacokinetics. Buprenorphine´s serum half-time in rats is estimated to be 2.8 hours although its effects can extend up to 8 hours in this species, due to existence of an extensive enterohepatic circulation (Gades et al., 2000; Othani et al., 1994). Finally, a study which measured the levels of CS in rats submitted to jugular catheterization which had

buprenorphine administration, showed results similar to our findings (Goldkuhl et al., 2010). In this latter study buprenorphine was also administered through SC route, at the same dosage and also 30 minutes before induction of general anesthesia. Buprenorphine induced an increase in CS levels, whose peak coincided with buprenorphine´s highest serum concentrations. In addition, the increase in CS levels was very similar to the one we found in our study.

How buprenorphine could have increased ACTH and CS levels remains unclear, but it may be related with its particular pharmacology. It is known that ACTH release is modulated by endogenous and exogenous opioids through action in several opioid receptors (Vuong et al., 2010; Gomez-Flores et al., 2000). The nature of this modulation varies with species, type of opioid (in particular its specific opioid receptor agonist/antagonist activity), dosage and route of administration (Leggett et al., 2009; Calogero et al., 1996; degli Uberti et al., 1995; Iyengar et al., 1987). As described before, most available literature does not describe an increase in CS levels following buprenorphine treatment and those which did it, did not clarify the underlying mechanism. Consequently, our hypothesis to explain our findings will be based in what is known about the effects of other opioids in HPA activity. The stimulation of the opioid μ and δ receptors has been associated to increased ACTH levels (Calogero et al., 1996; degli Uberti et al., 1995). Thus it may be hypothesized that buprenorphine increased ACTH levels through its μ and/or δ agonist effects. In rats, it is also known that the endogenous NOP system is involved in the acute HPA axis response to stressful stimulus (Delaney et al., 2012; Leggett et al., 2009). However, there are also reports demonstrating that the administration of nociceptine does not lead to CS elevation in mice (Prince-Zullig et al., 2009). Thus, buprenorphine´s NOP agonistic activity might have also played a role in our findings although this remains uncertain.

Alternatively, increased CS levels could have resulted from another mechanism than the action of ACTH on adrenal cells. One possibility might have been a direct effect of buprenorphine in adrenal cells. This has been reported with morphine and other opioids through effects in μ and κ receptors present in the adrenal´s *zone glomerulosa* cells (Kapas, Purbrick & Hinson, 1995).

On the other way round, it might be conceived that buprenorphine somehow facilited isoflurane´s capacity to increase HPA activity. In fact, in rats isoflurane was associated to increased CS levels when administered for long periods (Altholtz et al., 2006). How isoflurane exerts this effect is still unclear, but the mechanism may be specie-specific. In anesthetized rabbits isoflurane increases intra-operatively CS but not ACTH levels (Gil, Silván & Illera, 2007). In contrast, in mice anesthetized with isoflurane and administered pre-operatively with dexamethasone to prevent ACTH secretion, CS levels decrease, implying a role for ACTH in isoflurane modulation of CS levels in this specie (Jacobsen et al., 2012). In

Humans there is also conflicting evidence for and against a stimulatory effect of isoflurane in the HPA axis (Lattermann et al., 2003; Delogu et al., 1999). To our knowledge no study has investigated if buprenorphine interacts with isoflurane to increased ACTH and CS levels but this remains a possibility which cannot be discarded.

Our correlation analysis found a negative correlation between ACTH and MAP in G1 animals. This corroborates what is known about the relation of the HPA axis with decreased blood pressure (Darlington, Shinsako & Dallman, 1986). However, we believe that the role of low blood pressure in increasing ACTH and CS release in our findings was unlikely, as G1 had always higher MAP than G0.

How can our findings of buprenorphine-associated increased HPA activity be conciliated with the commonly accepted view that buprenorphine does not affect or decreases the post-operative HPA response? We believe that they can if some assumptions are taken in account. Most studies about the effects of buprenorphine on CS levels concerned its use as analgesic in the post-operative period. Because at this stage, pain is one of the most potent stimuli of HPA axis´s activation and buprenorphine is a good analgesic, it might be expected that its effects in mitigating pain would become predominant over other endocrine effects. In addition, our measurements were made intra-operatively and consequently our findings may be the result of the interaction between buprenorphine and isoflurane. Besides the different studies varied in factors such as drug dosages, route of administration and other experimental factors (e.g. nature of injury) which can also explain the differences in the effects of buprenorphine (Goldkuhl et al., 2010; Franchi et al., 2007; Gomez-Flores et al., 2000). Finally in light of the complex and still incompletely understood pharmacology of buprenorphine, our findings may not be that unexpected and can reflect a previously unknown mechanism. A similar example was provided by a previous cited study (Wilson, Ripsch & White, 2016) where buprenorphine unexpectedly increased tactile hyperalgesia and CS levels.

5.1.2. Effects of buprenorphine in metabolic variables

As stated before, G1 animals had significantly changes in metabolic parameters when compared with G0. Because the levels of PCO_2 did not differ significantly between both groups, it is reasonable to assume that these changes were most likely the result of an effect of buprenorphine (direct or indirect) in the regulation of the acid-base status by the kidney. To our knowledge there are no studies which have demonstrated a direct effect of buprenorphine or other opioids in the kidney´s ability to regulate acid-base status. Buprenorphine is actually considered one of the opioids of choice for use in patients with

kidney failure, because it is not associated to significant changes in kidney function (Davis et al., 2012).

Several hypotheses can be advanced to explain our findings. One is that buprenorphine directly increased renal HCO_3^- reabsorption and/or H^+ secretion. This would lead to an increase in HCO_3^- levels and consequently of BE and pH. However, although opioid receptors have been found in kidney tubular cells, it is currently believed that they do not play any role in renal ion transport (Hatzoglou et al., 1996). A buprenorphine-mediated increased in intestinal HCO_3^- reabsorption also seems unlikely, because opioids have been reported to induce the opposite effect (McKay, Linaker & Turnberg, 1981).

Alternatively, buprenorphine could have induced these metabolic changes through indirect mechanisms. One of this could have been the increase in ACTH and CS. ACTH administration has been associated to metabolic alkalosis in rats by increasing the relative rates of renal tubular H^+ secretion and Cl^- reabsorption (Kildeberg, Wamberg & Engel, 1988). ACTH also increases the gastrointestinal absorption of non-metabolizable bases (Wamberg, Engel & Kildeberg, 1983). Besides, in rodents CS is associated to renal proximal and distal tubular acidification and bicarbonate reabsorption (Malnic et al., 1997; Damasco et al., 1983). Against this hypothesis we have the fact that we were not able to find any type of correlation between ACTH and CS and pH, HCO_3 or BE. Finally, buprenorphine might have increased aldosterone secretion and/or action in the kidney tubules. This would lead to increased renal tubular H^+ secretion and consequently increased HCO_3^-, BE and pH. This hypothesis is supported by the fact that other opioids were shown to induce aldosterone secretion by rat adrenal cells, an action which seems to be mediated through μ-receptors (Kappas, Purbrick & Hinson, 1995). To our knowledge no studies have described the effect of buprenorphine in aldosterone secretion in rats. Because we did not measure aldosterone this hypothesis remains unproven.

Buprenorphine-treated animals had also significantly higher levels of PO_2 than G0 at T0, T1 and T2. The reasons for these findings were not determined. Several studies performed in mechanically ventilated dogs and rats have demonstrated that buprenorphine does not change significantly PO_2 levels (Gueye et al., 2001; Martinez et al., 1997). An improvement in respiratory mechanics is also unlikely, because buprenorphine does not seem to induce changes in airway dynamics (Ohya et al., 1993). Thus, the reason for this finding remains unknown and should be investigated in future studies.

5.1.3. Effects of buprenorphine in hemodynamic variables

When compared with G0, animals from G1 had higher MAP values at T0, T1 and T2 and lower HR at T0 and T1. Buprenorphine has been shown to influence these parameters in

previous studies (Ilbäck et al., 2008; Criado et al., 2000). In conscious rats, the administration of buprenorphine by SC injection led to an increase in HR and MAP in a dose-dependent manner (Ilbäck et al., 2008). In contrast Criado et al. (2000) found that in rats anesthetized with isoflurane, the administration of buprenorphine by IV injection led to the opposite effects (Criado et al., 2000). In dogs buprenorphine was also associated to decreased HR and MAP (Martinez et al., 1997). Therefore, the cardiovascular effects of buprenorphine seem to vary accordingly to specie, dosage, route of administration and presence of general anesthesia. How buprenorphine induces these effects is still incompletely understood. It is known that buprenorphine can increase cardiac contractility and SVR (Ilbäck et al., 2008; Martinez et al., 1997) and has direct electrophysiological effects (Boachie-Ansah et al., 1989). Based in this, we hypothesize that the increased MAP in G1 resulted from both increased cardiac contractility and SVR and the HR changes from its modulation of heart electrophysiology.

5.1.4. Effects of buprenorphine on apoptosis

Interestingly buprenorphine-treated animals had a higher apoptotic index in adrenal cortical cells than G0 animals. The pro-apoptotic effect of opioids has already been demonstrated in several cell types but especially in lymphocytes and splenocytes. These seem to depend from opioid ligation to opioid receptors in immune cells (Wang et al., 2002; Yin et al., 2000). However, to our knowledge the influence of buprenorphine or other opioids in apoptosis of adrenal cells has not been published before. In recent years, several studies have demonstrated that buprenorphine has pro-apoptotic effects in several cell types, including nerve (Kugawa, Arae, Ueno & Aoki, 1998) and tumor cells, including human lung carcinoma A549 and breast cancer cell line MCF-7 cells lines (Kugawa, Matsumoto & Aoki, 2004; Yoshida, Tokuyama, Iwamura & Ueda, 2000). The mechanisms on how buprenorphine induces apoptosis are incompletely understood. Nevertheless, they include increased caspase-3-like activity, increased mitochondrial permeability and activation of key molecules of mitochondrial apoptotic pathway including cytochrome c, caspase-9, caspase-7, and caspase-6, and increased expression of genes associated with polyubiquitination of apoptotic cellular protein (Kugawa & Aoki, 2004; Kugawa, Matsumoto & Aoki, 2004; Kugawa, Nakamura, Ueno & Aoki, 2004; Yoshida et al., 2000). An alternative hypothesis is that buprenorphine might have increased apoptosis through indirect mechanisms and especially by promoting the increase in CS levels. As it will be discussed later, increased CS levels can be pro-apoptotic in adrenal gland cells.

5.2. Analysis of G2

5.2.1. Hormonal variables

As expected in the animals of G2, HS was associated to an increase in HPA axis activation (Gundersen et al., 2003), manifested by a rise in ACTH and CS concentrations. In fact, from T0 to T1 ACTH increased 9 times and CS 2 times their original levels, which is in accordance to what has been described in the literature (Rushing et al., 2006; Molina & Abumrad, 1999). HS is known to induce HPA activation through several mechanisms including hypotension, hypoxemia, and by increasing the levels of several inflammatory mediators (such as cytokines) and hormones (Herman et al., 2016). We did find a positive correlation between ACTH and CS with lactate, ACTH with TNF-α and IL10 and a negative correlation between ACTH with BE and HCO_3. Thus, this corroborates the hypothesis in G2 HPA activity was triggered, at least in part by metabolic and cytokine changes. Our findings are similar to what has been described in other experimental models of HS (Rushing et al., 2006; Molina & Abumrad, 1999).

Contrarily to G3 animals, which will be discussed later, in G2 the normal feedback mechanisms of the HPA axis, which regulate and interconnect ACTH and CS secretion seemed to be preserved. For example, the high peak of CS at T1 was followed by a decrease in ACTH levels from T1 to T2, suggesting an inhibitory effect of CS in ACTH secretion at the pituitary and hypothalamic levels. It is known that glucocorticoids can inhibit hypothalamic CRH and ACTH secretion in minutes, through nongenomic mechanisms (Uchoa, Aguilera, Herman, Fiedler, Deak, de Sousa, 2014). The opposite occurred when CS levels decreased from T2 to T3, which was followed by an increase in ACTH levels. The positive correlation found between ACTH and CS also argues in favor that the normal regulatory and negative feedback between ACTH and CS was maintained.

There is however one aspect where our results differed from what has been described in other studies: the levels of CS in the post-HS period, especially towards the end of the experiment, were lower than what was previously described. (Rushing et al., 2006) . CS levels were not indicative of CIRCI, accordingly to the current guidelines (Marik et al., 2009). Nevertheless they were actually lower to those which were were considered as indicative of an inadequate adrenal response to HS in other studies (Stein et al., 2013; Rushing et al., 2006). In addition, levels of glucocorticoids similar to those we found were associated to poor outcomes in Human patients with HS(Stein et al., 2013). It was also evident that following the rise of ACTH levels from T2 to T3, a correspondent increase in CS secretion did not occurred. These observations thus suggest that in G2 animals, in T2 and especially T3, somehow the adrenal gland was less responsive to the stimulus of ACTH.

Several explanations can be advanced to explain these findings including:

- HS model was only of mild severity;
- Animals were under general anesthesia;
- HPA axis dysfunction;
- Presence of inhibitors of steroidogenesis (corticostatins).

These explanations will be discussed briefly in the following sections.

The low CS levels were due to the mild severity of HS

In this possibility, the lower levels of CS resulted from the fact that we used a model of HS which can be classified of mild severity (Hoppen et al., 2005). In fact, the degree of ACTH and CS increase after HS is related with the severity of the decrease in blood volume and MAP (Grässler, Jezová, Kvetnanský & Scheuch, 1990). Therefore by inducing a mild decrease in MAP and blood volume, our model was associated to a lower degree of HPA axis activation. It also allowed the body to more easily compensate HS and to return to the steady state. This would remove the need for maintaining the stress response and consequently CS levels would decrease. We believe that this hypothesis can explain part but not totally our findings. In fact, animals of G2 had a progressive worsening of several parameters, including hemodynamic (especially MAP) and metabolic parameters. These certainly would imply the need for an increased HPA activity. Therefore we believe that other factors played a role in our findings, in particular from T2 to T3.

The low CS levels were due to the use of general anesthesia

In this model we used anesthetized animals. This contrasts with other studies where HS was induced in conscious animals (Molina & Abumrad, 1999). It is possible that by using general anesthesia, the response of the HPA axis to HS was suboptimal. Consequently this would lead to the secretion of lower levels of CS. The influence of general anesthesia in the response to HS has been recognized since the second half of the XX century (Theye, Perry & Brzica, 1974). General anesthesia can influence the response to HS through several ways including by a direct modulation of the pituitary-adrenal response (Cai, Deitch & Ulloa, 2010), although highly dependendt from the type of anesthetic (Mizutani et al, 1998; Adams et al, 1991). Although it is true that anesthetized animals have low CS levels than conscious animals, and we cannot rule out that general anesthesia might played a role in our findings, we believe that it was not the principal factor for the results. This is because it was not able

to block the response to hypovolemia and also because it did not seemed to affect the HPA response in G0 and G1.

The low CS levels were due to the of HPA axis dysfunction

Another possibility is that G2 animals, developed a dysfunction in the HPA axis. The presence of adrenal dysfunction following HS has been previously demonstrated in both experimental and clinical settings (Rushing et al., 2006; Hoen et al., 2002; Wang et al., 1999). If this was the case, what could have caused it? It is unlikely that the low CS levels resulted from a dysfunction at the central level. As discussed previously, the levels of ACTH were in accordance to what has been described in the literature and the ACTH response to changes of CS levels seemed normal. Thereforeit most probably resulted from a dysfunction at the adrenal level. In turn, this might have been caused by several etiologies:

- Development of adrenal necrosis;
- Development of adrenal apoptosis;
- Development of adrenal dysfunction due to systemic and local inflammation;
- Presence of corticostatins in plasma.

The two first hypotheses will be more thoroughly discussed in the analysis of histopathological variables of G2. The remaining two hypotheses will be described below.

Adrenal dysfunction caused by increased inflammation

HS is associated to an overwhelming production of cytokines such as TNF-α, IL6 and IL10 (Roumen et al., 1993; Ayala et al., 1991; Bitterman et al., 1991). The effects of these cytokines in adrenal function are complex. Cytokines can interfere in adrenal function by acting at a central (e.g. interfering with ACTH secretion and release) or at a local level. To further add complexity to this issue, the effects of cytokines in steroidogenesis vary between studies and depend from factors such as type of cytokine, if the cytokine is tested in adrenal cell culture, tissue or in intact animals and, in case that steroidogenesis is evaluated in cell culture, the specie of origin of adrenal cells.

Systemic high levels of inflammatory cytokines were initially shown to depress the magnitude of cortisol response to corticotropin (Catalano, Parameswaran, Ramachandran & Trunkey, 1984). It is now known that some cytokines increase steroidogenesis by both direct effects at

the adrenal gland and indirectly, by stimulating ACTH secretion (Bornstein, Rutkowski & Vrezas, 2004). It was shown that TNF-α, IL1 and IL6 increase steroidogenesis by stimulating ACTH release (Bornstein, Rutkowski & Vrezas, 2004). Nevertheless, increased levels of IL6 have also been associated to development of CIRCI in some studies (Hoen et al., 2002).

It is also known that intra-adrenal cytokines are important for the normal physiology of the adrenal gland. Within the adrenal, macrophages and lymphocytes, normally present in the adrenal cortex, and adrenocortical and chromaffin cells produce cytokines, such as IL1, IL6, TNF-α, leukemia inhibitory factor (LIF), and IL18 which have a key-role in the immune-adreno-cortical communication (Bornstein, Rutkowski & Vrezas, 2004). This is illustrated by the fact the both adrenocortical and adrenomedullary cells are provided with specific receptors for IL1, IL2, and IL6 (Nussdorfer & Mazzocchi, 1998). In adrenal tissue, TNF-α has been reported to have both stimulatory (Mikhaylova et al., 2007; Darling, Goldstein, Stull, Gorschboth & Norton 1989) and inhibitory effects (Jaattela et al.1991; Jaattela et al. 1990). In contrast, IL6 is mainly recognized to have a stimulatory effect in glucocorticoid synthesis (Nussdorfer & Mazzocchi, 1998; Päth et al., 1997; Gadient et al., 1995).

There is also evidence that increased production of intra-adrenal cytokines is pivotal for an adequate adrenal response to inflammatory and immune challenges and stresses (Jennewein et al., 2016; Yu et al., 2016; Nussdorfer & Mazzocchi, 1998). For example, TLR-$2^{-/-}$ rats submitted to LPS administration, have not only decreased TNF-α, IL1 and IL6 systemic responses but also decreased expression of adrenal TNF-α, IL1 and IL6 receptors. In addition the latter was associated to their decrease in the systemic levels of CS (Bornstein et al., 2004).

However, in conditions associated to systemic high levels of cytokines such as HS, the cytokine “storm” can have other effects rather than a direct influence in steroidogenesis. It is known that increased cytokine levels can cause direct tissue injury including in the the adrenal gland (Cai, Deitch & Ulloa, 2010). Furthermore, a recent study found that the administration of pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide with anti-inflammatory properties, improved the adrenal dysfunction caused by CLP and CLP associated to etomidate, by modulating lymphocyte function and decreasing systemic inflammation (Liu et al., 2016). Systemic inflammation also increases the expression of local adrenal pro-inflammatory mediators by parenchymal immune cells, such as resident macrophages, leading to increased intra-adrenal inflammation (Kanczkowski et al., 2013a; Kanczkowski et al., 2013b; Kanczkowski et al., 2013c; Hu et al., 2009; Engström et al., 2008). This increased intra-adrenal inflammation, with increased intra-adrenal cytokine activity has been linked to adrenal dysfunction and even CIRCI in several experimental

models of sepsis (Jennewein et al., 2016; Wang et al., 2015; Kanczkowski et al., 2013a; Kanczkowski et al., 2013c; Hu et al., 2009).

In our study adrenal inflammation and intra-adrenal cytokine levels were not quantified. In addition no correlation was found between cytokine and CS levels. Thus, the hypothesis that HS-associated adrenal inflammation, induced and/or amplified by systemic inflammation contributed to the decreased CS levels of G2 remains unproven.
In G2, the only correlations which were found between cytokines and hormones of the HPA axis were the positive correlations between TNF-α and IL10 with ACTH. This corroborates what has been described in the literature, and most probably reflect their stimulatory activity of the the HPA axis (Dunn, 2000; Smith et al., 1999).

The low CS levels were due to the presence of inhibitors of steroidogenesis (corticostatins)

In recent years, substances known as corticostatins which inhibit steroidogenesis have been described in several settings (Tominaga et al., 1990). To our knowledge their presence in HS has not yet been described. It is also possible that increased corticostatins levels influenced adrenal gland function in G2. However because their levels were not measured we cannot confirm this hypothesis.

5.2.2. Immunological variables

When compared with animals of G0 and G1, G2 animals had significantly higher levels of IL10. This was an expected finding, because increased IL10 levels have been found as early as 2 hours following trauma and HS (Schneider et al., 2004, Neidhardt et al., 1997). In G2, a positive correlation was found between the levels of TNF-α and IL10. This is in accordance to what has been found in the literature, because TNF-α has been showed to induce IL10 release (Brøchner & Toft, 2009). IL10 levels were also negatively associated with BE and HCO_3 levels. The significance of this finding is unknown. There are studies which have associated IL10 levels to changes in acid-base status. For instances, the levels of IL10 were higher in patients with diabetic ketoacidosis which presented a pH < 7.2 than in patients with higher pH (Karavanaki et al., 2011). By contrary, the exposure of LPS-stimulated RAW 264.7 cells to two forms of acidosis (lactic and hyperchloremic acidosis) was associated to decreased IL10 levels (Kellum, Song & Li, 2004). Alternatively, the negative correlation of IL10 with these metabolic parameterscould have been a reflection of increased tissue hypoperfusion. In this line of reasoning, increased tissue hypoperfusion increased tissue injury, wich led to subsequent increases in TNF- α production and DAMPs release, which

are known stimulators of IL10 release (Maslanik et al., 2013; Brøchner & Toft, 2009; Levy et al., 2007).

The lack of significant increases in TNF-α and IL6 was unexpected, because it has been demonstrated that HS is associated to increases in the levels of these cytokines, in both experimental (Molina & Abumrad, 1999; Ayala et al., 1991; Ayala et al., 1990) and clinical studies (Roumen et al., 1993). Several reasons may be advanced to explain these findings and these will be presented below:

- *Presence of high levels of glucocorticoids:* This hypothesis will be discussed with more detail in next section, because the lack of CS was the most likely explanation for the high cytokine levels of G3;

- *The mild degree of severity of HS*: Several studies have demonstrated that the magnitude and duration of the post-HS inflammatory response correlates with the degree and duration of oxygen debt (Sato et al., 2010; Bitterman et al., 1991). Based in the amount of blood which was collected, our model of HS was considered of mild severity (Hoppen et al., 2005). In addition, the duration of HS was just of one hour. Thus the degree of oxygen debt which was induced might have not been severe enough to induce a pronounced increase in TNF-α and IL6 levels when compared with other studies. The problem with this hypothesis is that despite thelow severity , in our model there was evidence that HS promoted hypoperfusion severe enough to increase oxygen debt, manifested by the increased lactate and the decreased HCO_3 and BE). Thus, although the mild severity of the model may partially explain the low cytokine levels, it was probably not the only factor which contributed to these findings.

- *Increased cytokine levels mainly occurred at a local/tissue level:* HS increases both local and systemic levels of cytokines and these are not correlated with each other (Angele, Schneider & Chaudry, 2008; Molina et al., 1997). In our study it is possible that cytokine production occurred mostly at a local level, and the levels which were achieved were not sufficient to reach the systemic circulation. Because we did not measure tissue cytokine levels this hypothesis remains unproven.

- *Differences in experimental models*. Part of the discrepancy observed between our results and the ones of other studies may be a reflection of differences in experimental methodology. For example, a recent study demonstrated that volume-controlled and pressure controlled models of HS differ in the amount of cytokine production (Pfeifer et al., 2013). Another example is that in some models, the combination of trauma and HS blunts the systemic release of TNF-α and IL10 and that their kinetics do not reflected the magnitude of the physiological response or the

severity of the overall insult (Baker et al., 2012; Namas et al., 2009). The results of these studies support the concept that major trauma or the combination of polytrauma with HS in the experimental setting induces a state of immunoparalysis which is manifested by aninability to mount an appropriate cytokine response in the systemic circulation (Hotckiss et al., 2003). It is unlikely that this occurred in our model, because surgical intervention for catheter placement is considered a minor trauma with a limited role in cytokine activation. In fact experimentalmodels such as our own, HS ischemia and I/R injury and not trauma are considered the main drivers of immune system activation (Schimtz et al., 2010).

- *The time of blood sampling may have precluded the ability to detect increased cytokines levels*: Because the half-life of most cytokines is very short, it is possible that at the time of sampling, cytokines levels could be already under the limit of detection. This hypothesis is particular applicable to to TNF-α, which has a serum half-life of only 20 minutes (Ferguson et al., 1997). In addition, studies have shown that the increase in TNF-α levels which occur after HS is of short duration (Rhee et al., 1993; Ayala et al., 1990). Studies have shown that TNF-α levelspeak around 30 minutes after HS and then start to decrease, becoming undetectable several hours later (Rhee et al., 1993; Ayala et al., 1990). In our experiment, because the first blood sampling post-HS was taken at 90 min, it is possible that at this time, the peak in TNF-α was missed. The issue of TNF-α´s short half-life is even more relevant in the context of our HS model of mild severity, which is expected to induce only modest increases in TNF-α. Therefore it can be hypothesized that the combination of a short half-life associated to a modest increase in TNF-α levels contributed to make its detection even more difficult. Also in IL6, the time of sampling could have also interfered with its measurements, but in this case in a different way. Because IL6 reaches its peak levels around 4-6 hours post-HS (Sapan et al., 2012; Liu et al., 2007; Roumen et al., 1993), in our model what might have happened was that the experiment didn´t last long enough to detect its highest levels.

- *Influence of the place of sampling*: Several experimental models of HS have demonstrated that there is a temporal discrepancy between the levels of TNF-α and IL-6 obtained from different vascular beds, with cytokine levels increasing first in the portal bloodstream and only after in peripheral blood (Liu et al., 2007). Because we only measured cytokine levels in the peripheral circulation if the same happened in our model remains unclear.

- *Increase in anti-inflammatory mediators*: The contribution of CS has already been discussed. The increase in IL0 levels could have also contributed to the decrease in

TNF-α levels. In addition increased systemic levels of IL6 also possess an anti-inflammatory role. Consequently IL6 may acted in combination with IL10 to limit TNF-α production (Tilg, Dinarello & Mier, 1994).

5.2.3. Metabolic variables

In G2 HS led to several metabolic changes, including an increase in lactate and a decrease in BE and HCO_3 levels. These findings are in agreement in what has been described in the literature. HS is associated to the development of metabolic acidosis, which becomes manifest as early as 12 minutes after hemorrhage onset (Torres Filho et al., 2010). Part of this metabolic acidosis results from increased activity of systems associated to anaerobic energy production. This are activated after the decrease in aerobic metabolism which results from thedecrease in blood volume, CO and tissue DO_2 (Torres et al., 2004; Chang et al., 2000; Tung et al., 1976). In the initial stages of HS, metabolic acidosis also results from the phenomenon of dilutional acidosis, as the compensatory mechanisms to HS begin to operate. During the initial phase of blood volume restoration, to compensate the volume lost with hemorrhage, extravascular fluid which is low in protein content moves into the vascular space. This leads to hemodilution, decreasing plasma osmolality and protein and Na^+ concentrations. The decrease in Na^+ concentrations is associated to acidosis (Alfaro, Pesquero & Palacios, 1999).

Interestingly, and although at T0, animals of G2 were already acidotic when compared with G0 and G1 (but not in a statistically significant way), HS development was not associated to a further decrease in pH levels. The most likely reason for the maintenance of systemic pH was the use of mechanical ventilation which continuously adjust PCO_2 levels. Interestingly, experimental studies have demonstrated that maintaining effective ventilation is actually pivotal for achieving a normal blood pH in HS (Torres Filho et al., 2010; Alfaro et al., 1999).

The decrease in PO_2 levels after HS was also described in other models and was expected (Rose, Kheirabadai & Klemcke, 2013; Torres Filho et al., 2010). Interestingly, and contrary to what was found in G3, after T1, PO_2 levels remained stable until the end of the experiment. As it will be discussed in the G3 section, this difference most likely resulted from the development of lung injury in G3.

The metabolic changes which were found in G2 were moderate in severity, which was expected considering with the modest state of hypovolemia. It is known that at levels of MAP of around 35-45 mmHg, the body´s compensatory mechanisms to hemorrhage are still effective, minimizing the changes induced by HS (Rönn, Lendemans, de Groot & Petrat, 2011). Our results suggest that the body was able to compensate in part the development of hypovolemia, as confirmed by the decrease in lactate levels, especially after resuscitation. Nevertheless, metabolic indicators never returned to pre-HS levels. In addition some of the

metabolic changes actually got worse from T2 to T3, implying that tissues remained hypoperfused, most probably due to a progressive worsening of global hemodynamics (see below) and microcirculatory disturbances.

5.2.4. Hemodynamic variables

Even considering that the animals of G2 began with high blood pressures, as expected the development of HS induced a marked decrease in MAP from T0 to T1. Nevertheless at the end of the period between T0 and T1, MAP levels were still in the normal range, suggesting that at this stage, the body´s compensatory mechanisms to hypovolemia were effective. This is in accordance with what was described inthe literature (Rönn et al., 2011). However, from T1 to T3, MAP continuously decreased. We hypothesized that this decrease was due to a state of vascular decompensation which typically occurs in late phases of HS. Although the mechanisms of this condition are still being elucidated, studies have shown that this is related with desensitization of adrenergic receptors, membrane hyperpolarization of vascular smooth muscle cells, desensitization of the vascular musculature to Ca^{2+}, effects of acute-phase cytokines such as TNF-α, IL1 and IL6 (especially at the early stages of the process) and also of increased levels of NO and endothelin (at later stages) (Liu & Dubick, 2005; Liu, Ward & Dubick, 2003). As mentioned previously, CS levels were also lower than expected. Although their low levels were not sufficient to make the diagnosis of CIRCI, we hypothesize that the decrease in glucocorticosteroid levels might have contributed to the worsening of blood pressure. The role of glucocorticosteroids in the hemodynamics of HS will be more thoroughly discussed in the G3 section.

Alternatively, the decrease in MAP could have resulted from a decrease in cardiac function. Cardiac dysfunction might have occurred with metabolic acidosis, increased local cytokine activity and microcirculatory dysfunction. The fact that the decrease in MAP was more pronounced after resuscitation raises also the hypothesis that I/R injury played a role in this process, although this remain unproven.

Finally, it is important to note that control animals (G0 and G1) also displayed a continuous, although less marked, decrease of MAP from T0 to T3. This suggests that other factors rather than HS influenced the dectrease in MAP. The most likely factor was the use of isoflurane anesthesia.It has been show that isoflurane decreases MAP in rats, mainly by decreasing SVR (Seyde, Durieux & Longnecker, 1987).

5.2.5. Molecular biology variables

A large variation was found in the results of molecular biology variables. The only statistically significant differences were found between in *IL6* between G0 with G2 at T0, and in *IL10* between G0 and G2 at T1. In both cases the genetic expression of the cytokines was found to be lower in G2. The explanation for these findings remains unknown. Nevertheless, the limited number of samples with enough mRNA levels and the high variability of the results limit our data interpretation. Because more significant differences were found between G3 and the other groups, this section will be discussed more in detail in the section of G3 animals.

5.2.6. Histological variables-apoptosis and necrosis

As described previously, adrenal gland necrosis and apoptosis were suggested as possible causes of adrenal dysfunction in G2. This and other aspects will be discussed below.

Adrenal gland necrosis as a cause of adrenal dysfunction

Our first hypothesis for the cause of adrenal dysfunction is adrenal necrosis. In this hypothesis HS led to necrosis of adrenocortical cells, which in turn decreased the production and secretion of steroid hormones. In our study, we did find necrosis in the adrenal gland whose score was similar to the one reported in another experimental study (Rushing et al., 2006). In addition this necrosis was more prominent in the inner part of the adrenal cortex. The existence of adrenal necrosis following HS has been described in several studies. Kajihara et al. (1977) first demonstrated in a canine model that HS leads to necrosis of the adrenal medulla. The same authors in a subsequent study also confirmed that the same occurs in the adrenal cortex (Kajihara et al., 1983). The latter study provided the first detailed description of the pathological changes that occur in the adrenal after HS. In their model, Kajihara et al. (1983) reported that the first pathological changes were present 1 hour after hemorrhage and consisted in the sticking of leukocytes to the adrenal vessel´s sinusoidal wall. This was followed by the appearance of irregular masses of fibrin strands in the sinusoidal space, development of large gaps in the sinusoidal wall and leukocyte infiltration of *zona fasciculata* and *reticularis*. Adrenocortical cell´s than began to develop intracellular degenerative changes, which included aggregation and dilatation of the smooth endoplasmatic reticulum and intracellular edema. As HS progressed, the degenerative changes became more severe. Small necrotic foci, hemorrhage and inflammatory infiltration

become apparent in several areas of the gland, but especially in the inner half of *zona fasciculata* and *zona reticularis*. By contrast *zona glomerulosa* was relatively spared. After resuscitation the destruction of cordal structure, hemorrhage and inflammatory infiltration became even more severe. Towards the end of the experiment, when the terminal stage of HS was reached, the pathological lesions reached its maximum. Interestingly, the authors found that the terminal stage was accompanied by a marked decrease in cortisol secretion. In another study Rushing et al. (2006) also found evidence of adrenal necrosis in a rat model of HS. In this study too, adrenal necrosis was associated to the development of relative adrenal insufficiency in the animals submitted to HS.

The association of adrenal necrosis to adrenal dysfunction has also been identified in other critical conditions including post-surgery (McNicol et al., 2014; Cramer et al., 1994; Siu et al., 1990) and sepsis. In sepsis, the development of adrenal necrosis and hemorrhage, with extravasation of red blood cells and leukocytes (Siegel, Grinspoon, Garvey & Bilezikian, 1994), is considered one of the main causes of adrenal insufficiency for more than one century (Friderichsen, 1918; Waterhouse, 1911). In fact, it is so common that is known as the Waterhouse-Friderichsen Syndrome. Adrenal hemorrhage secondary to sepsis typically occurs in meningococcemia and other disseminated bacterial infections. Contributing factors for it include platelet aggregation followed by venous thrombosis; vasoconstriction and disruption of the vascular endothelium; and enlargement of the adrenal glands due to a combination of decreased venous drainage and sepsis-associated increased arterial blood flow (Jung et al., 2011). All these factors combine to causeintramedullary ischemia and vessel damage. When reperfusion occurs, bleeding frequently ensues (Egan et al., 2009).

Destructive changes in the adrenal cortex and medulla have also been described in experimental models of endotoxic shock (Bardakhch'ian et al., 1986). A study found that the adrenals of patients who died with septic shock had higher levels of hemorrhage and necrosis than the adrenals obtained from patients who died from other conditions (Polito et al., 2010). Inflammation, necrosis and hemorrhage of the adrenal cortex were also more predominant in *zona fasciculate*, as it what found in HS models,. In addition sepsis was associated to depletion of lipid droplets in cells of *zona fasciculata*. Because lipid droplets store cholesteryl esters which are the substrate for steroidogenesis in adrenal gland cells (Gwynne & Mahfee, 1989), Polito et al. (2010) suggested that lipid droplet depletion can contribute for adrenal insufficiency in sepsis (Polito et al., 2010). Interestingly, in the same study mice submitted to CLP or LPS administration had also lipid depletion but no evidence for necrosis or hemorrhage in the adrenals. This finding highlights that adrenal necrosis in sepsis may be specie dependent or related with the experimental conditions.

The literature than supports that adrenal necrosis could have been a contributing factor to development of adrenal dysfunction in G2. If this was the case, what were the mechanisms

associated to HS that led to adrenal necrosis in these animals? One obvious explanation is ischemia, as proposed by Rushing et al., (2006). In fact a positive correlation was found between the development of adrenal necrosis and one indicator of tissue hypoperfusion: BE.

The way how the adrenal gland receives its arterial blood supply is such that some anatomical areas are at higher risk of developing ischemia than others. It is known that more than 90% of the arterial blood to the adrenal cortex is firstly distributed to the *zona glomerulosa* and only afterwards to the inner cortical zones (Sparrow & Coupland, 1987). Consequently *zona reticularis* and *zona fasciculata* are at particular risk of suffering from ischemia. These were the areas more affected by necrosis in our study and in the study of Kajihara et al., (1983). furthermore, it is also known that blood supply to the zones of the adrenal gland is differently influenced by the presence of HS. During this condition, total adrenal blood flow does not decrease until MAP fells below 30 mmHg. However cortical blood flow is significantly reduced when MAP fells below 50 mmHg (Hamaji et al., 1986) and it is mainly directed to *zona glomerulosa* (Jasper et al., 1990). In contrast medullary blood flow is increased between 100-400% and only returns to baseline when MAP decreases below 30 mmHg (Hamaji et al., 1986). All these studies give support to our hypothesis that adrenal ischemia was the main cause of adrenal necrosis in our model, although this remains unproven.

Besides ischemia, it is also possible that adrenal necrosis was triggered by oxidative stress and adrenal inflammation. Increased cytokine levels such as TNF-α and ROS have been associated to cell necrosis (Galluzzi et al., 2012; Vanlangenakker et al., 2012; Zhang et al., 2009).Experimental studies have also demonstrated that adrenal inflammation occurs in illnesses associated to systemic inflammation such as sepsis and that it is associated to cell death and adrenal dysfunction (Jennewein et al., 2016; Kanczkowski et al., 2013c).

As described in the literature review, during HS an important source of ROS, increased cytokine levels and tissue inflammation is I/R injury. In the study of Kajihara et al. (1983), the authors reported that adrenal necrosis was particularly prominent after fluid resuscitation (Kajihara et al., 1983). Because I/R injury, by definition, occurs after tissue reperfusion, which in HS commonly occurs with fluid resuscitation, this observation suggests that adrenal necrosis was potentiated by I/R injury.

In recent years, several types of necrosis have been recognized. For a long time, necrosis has been regarded as an accidental and uncontrolled caspase-independent cell death. However, accumulating evidence has now revealed that some forms of necrosis actively involve defined intra-signaling pathways that contribute to the cellular demise, in a process similar to what occurs in apoptosis. An active form of necrosis has actually been recognized and termed necroptosis (Dondelinger et al., 2016). Necroptosis can be triggered by cytokines such as TNF-α, PAMPs and DAMPs (through TLR-3, -4 and -9), I/R injury, Ca^{2+} overload,

hypoxia, infectious agents, DNA damage, oxidative and nitrosative stress (Vanlangenakker et al., 2012; Degterev et al., 2005). The most distinctive biochemical marker of this type of cell death is its dependency on RIPK3 kinase activity (Vanlangenakker et al., 2012). Necroptosis has been recognized in experimental murine models of sepsis (Sharma et al., 2014; Duprez et al., 2011), traumatic brain injury (Wang et al., 2012), intra-cerebral hemorrhage (Chang et al., 2014) and non-alcoholic steatohepatitis (Afonso et al., 2015). The importance of this type of cell death has been shown in studies such as the one performed by Duprez et al., (2011). In this, mice *RIPK3*$^{-/-}$ and wild mice treated with necrostatin (a known inhibitor of necroptosis) which were submitted to sterile SIRS induced by TNF-α administration or sepsis by CLP model, had decreased tissue injury and mortality when compared to control animals. They also had decreased systemic cytokine and DAMPs levels (Duprez et al., 2011). TNF-α is one of the mediators known to induce directly necroptosis, in a process where ROS play a significant role (Schenk & Fulda, 2015). RIPK1 and RIPK3, the main modulators of necroptosis were recently identified to be regulators of inflammatory signaling (Silke, Rickard & Gerlic, 2015), which suggests that inflammation may also modulate the necroptotic pathway. The processes of apoptosis and necroptosis are also tightly interconnected, sharing several intra-signaling pathways (Chang et al., 2014). Necroptosis was not evaluated in this study but based in what has been published, it is possible that contributed to the adrenal necrosis found in G2 animals as well. Because I/R injury and tissue inflammation are associated to both necrosis (including necroptosis) and apoptosis, they will be more thoroughly discussed in the apoptosis section of G2 and especially in the G3 section.

Interestingly, we also found a positive correlation between the degree of necrosis and the levels of IL10. To our knowledge no study has described a direct effect of IL10 in inducing adrenal necrosis in rats or other species. However it is known that endogenous DAMPs (Maslanik et al., 2013) induce the secretion of IL10. Thus we hypothesize that this positive correlation can be explained by the following mechanism: adrenal necrosis led to increased release of DAMPs and these in turn, stimulated the secretion of high levels of IL10. The significance of this correlation to our results remains unknown.

Although the hypothesis that adrenal necrosis was associated to development of adrenal dysfunction in G2 animals is plausible and attractive, we were not able to find a correlation between the levels of ACTH and CS and the degree of adrenal necrosis. This may suggest that necrosis was not associated to adrenal dysfunction, contrasting with the findings of Rushing et al. (2006), where it was suggested as the main cause of CIRCI. Importantly this study differed from our one in significant aspects, which can explain the disparity of results. In the study of Rushing et al. (2006), general anesthesia was different (the authors used ketamine and xylazine), there was no mechanical ventilation, hypotension was milder (MAP

of 65 mmHg), the duration of HS was longer (4 hours) and resuscitation consisted in Ringer Lactate, administered for two hours. All these factors can have a different impact in adrenal blood flow and in the presence of adrenal inflammation, thus conditioning cell's fate after injury.

Alternatively, the lack of a significant correlation of our study may point out that necrosis was not the only factor that contributed to adrenal dysfunction in G2. We believe that this was the case. In fact we hypothesize that in addition to adrenal necrosis, adrenal apoptosis and inflammation played a fundamental role in adrenal dysfunction.

Adrenal gland apoptosis and adrenal gland dysfunction

In this study, HS was associated to development of adrenal gland apoptosis in both cortex and medulla. In addition G2 was the group which had the higher apoptotic rate. Curiously, the presence of apoptosis changed accordingly to the area of the adrenal gland, being higher in the cortex than in the medulla. Besides in the cortex was particularly frequent in *zona fasciculata* and *zona reticularis*. Because these areas are responsible for the production of glucocorticoids in the rat (Vinson 2016; Mitani, 2014), it is possible that adrenal apoptosis contributed to the development of adrenal dysfunction in G2.

Apoptosis is a genetically programmed mechanism of cell death, which is important for regulating many organ functions including embryo development and inflammation (Hattori et al., 2010). In normal adrenal glands, apoptosis has physiological functions in the development, renewal and remodeling of the adrenocortical structure (Pihlajoki et al., 2015). It also functions as a regulatory mechanism that allows the adrenal gland to cope with stresses and changes in functional demands (Bozzo et al., 2006).

Recently the contribution of apoptosis for development of MOF in several critical illnesses has been recognized (Thacker et al., 2013; Moran et al., 2009; Papathanassoglou et al., 2000; Rollwagen et al., 1998), especially in sepsis and traumatic brain injury (Bayir & Kagan, 2008). Apoptosis in sepsis has been particularly studied and it is now considered as pivotal in the pathophysiology of sepsis (Hattori et al., 2010). For instances, lymphocyte apoptosis has been considered one of the mechanisms behind the immunosuppression that occurs in sepsis and septic shock (Hsieh, Athar & Chaudry, 2009). In addition, delayed neutrophil apoptosis in septic patients contributes to its accumulation in vital tissues and to subsequent organ injury (Hsieh, Athar & Chaudry, 2009).

In HS and trauma there is also evidence, mainly experimental, that apoptosis occurs in several organs contributing to its dysfunction. This include organs such as the liver (Yang et al., 2011), small intestine (Lu et al., 2008), kidney (Moreira et al., 2016; Cotogni et al., 2010), heart (Zhou et al., 2015), spleen (Deng et al., 2016), thymus (Guan et al., 1998) and lung

(Barlos et al., 2008). Apoptosis has also been recognized as a pivotal mechanism in HS-associated increased microvascular permeability. The latter seems to be related with development of endothelial cell apoptosis (Sawant et al., 2015; Childs et al., 2006).

The occurrence of adrenal apoptosis has also been described in several critical illnesses, especially in the experimental setting, including trauma (Didenko et al., 1996), ANP (Yu et al., 2016; Yu et al., 2012) and sepsis (Liu et al., 2016; Wang et al., 2015; Kanczkowski et al., 2013c; Flierl et al., 2008). To our knowledge only one study described the occurrence of adrenal apoptosis in HS (Rushing & Britt, 2007). The presence of apoptosis was associated to development of adrenal dysfunction in some but not all of these studies. This was the case of study in HS (Rushing & Britt, 2007). Some of these studies will be described in more detail in the following sections.

Didenko et al. (1996) found apoptotic cells in the adrenal cortex of organ donors who had undergone severe trauma prior to death (Didenko et al, 1996). The same group, in a later study, evaluated for the presence of apoptosis and p21 (an indicator of DNA damage), in the adrenal glands of rats submitted to several types of injury (Didenko et al., 1999). The latter included ischemia induced by ligation of the renal artery followed by reperfusion, sepsis (CLP model), hypovolemia, ANP and administration of chemical agents (zymosan and acrylonitrile). All interventions increased p21 expression and DNA damage in adrenocortical cells, but apoptosis was infrequently detected. In 2008, Flierl et al., (2008) identified apoptosis in adrenomedullary cells of mice submitted to CLP and found that this was related with the complement anaphylaxin C5a (Flierl et al., 2008). Yu et al. (2012) demonstrated adrenocortical apoptosis in rats submitted to ANP. Interestingly, this study found that severer ANP was associated to a higheradrenal apoptotic rate and a lower level of CS(Yu et al., 2012). This led the authors to conclude that the decrease in CS was related to inflammation and apoptosis in the adrenal cortex. The work of Yu et al. (2012) was the first which associated adrenal apoptosis to CIRCI in a critical illness.

Kanczkowski et al., (2013a) demonstrated in mice that SIRS induced by LPS administration was associated to adrenocortical and chromaffin cell´s apoptosis. The authors showed that mice deficient for endothelial locus-1 (Del-1), an endothelial-derived anti-inflammatory factor which antagonizes leukocyte adhesion, had increased adrenal leukocyte accumulation, inflammation and apoptosis (Kanczkwoski et al., 2013c). Interestingly 24 hours post-LPS, the animals with higher degree of apoptosis had also reduced CS and ACTH levels. More recently a murine study suggested that in rats, part of etomidate-associated CIRCI could be related with development of adrenal apoptosis (Liu et al., 2015). In contrast with previous studies, adrenal apoptosis was not associated to adrenal insufficiency in a rat model of intra-abdominal hypertension (Akkapulu et al., 2015).

Finally two studies of 2016 provided solid evidence that adrenal apoptosis contributes to development of CIRCI in sepsis. The already cited study of Liu et al., (2016) found that PACAP, a pleiotropic neuropeptide with known antiapoptotic functions (Arimura, 1998), decreased adrenal apoptosis associated to CLP in rats. These effects of PACAP werein part associated to decreased inflammation (Liu et al., 2016). Yu et al., (2016) also demonstrated that PARP inhibition was associated to decreased adrenocortical apoptosis and adrenal insufficiency in a rat model of ANP.

Therefore, the majority of literature demonstrates that, adrenal gland apoptosis is a contributing factor to development of CIRCI in several critical illnesses. However to our knowledge, our study is the first which investigated the role of adrenal apoptosis in HS-associated CIRCI.

What might have caused adrenal apoptosis in the animals of G2

The occurrence of adrenal apoptosis in our model was not unexpected. In fact, critically illnesses such as HS affect the apoptotic rates in organ tissue cells and their respective endothelial cells through several mechanisms (Papathanassoglou et al., 2000). These include: release of inflammation-related cytokines; production of ROS associated with I/R injury and states of low tissue perfusion; expression and release of HSP from tissue cells and the liver; elevated glucocorticoid concentrations after adrenal cortex activation and release of bacterial products into the systemic circulation (Papathanassoglou et al., 2000).

The systemic pro-inflammatory state associated to HS could have been one of the mechanisms which led to adrenal apoptosis. Inflammation and apoptosis are tightly interconnected (Hattori et al., 2010) and increased levels of pro-inflammatory mediators have been associated to pro-apoptotic markers in critically ill patients (Papathanassoglou et al., 2003). Furthermore apoptosis could have also been induced by intra-adrenal inflammation.

If intra-adrenal inflammation is severe or prolonged in time, it can lead to adrenal dysfunction (discussed previously) and apoptosis (Yu et al., 2016; Kanczkowski et al., 2013a; Kanczkowski et al., 2013c; Yu et al*.,* 2012). The mechanisms on how adrenal inflammation triggers apoptosis are still incompletely understood. Nevertheless studies have shown that endothelial and local immune cell activation, and neutrophil adhesion to the endothelium followed by its influx into the adrenal parenchymal are main contributing factors (Jennewein et al., 2016 Wang et al., 2015; Kanczkowski et al., 2013c; Engström et al., 2008). I/R injury (Didenko et al., 1996), complement (Flierl et al., 2008), PARP (Yu et al., 2016) and NF-kB pathway activation (Rushing & Britt, 2007) have also been associated to the development of adrenal apoptosis.

Another mechanism which might have increased adrenal apoptosis in our study were the increased levels of several hormones with pro-apoptotic effects and which are known to be increased in HS. These include glucocorticosteroids (Almeida et al., 2007) and angiotensin II (Carsia et al., 1998).

In resume there are several hypotheses which explain why animals of G2 (and G3) had higher levels of adrenal apoptosis than G0 and G1. However, it was also evident that animals of G2 had higher cortical and medullary apoptotic indexes than etomidate-treated animals. The reason for this difference was not determined, although there are several possible explanations. These include differences in hormone and cytokine levels and the effects of etomidate itself and will be discussed in more detail in the G3 section.

Apoptotic index was different accordingly to the different areas

Despite the fact that adrenocortical and medullary apoptosis were positively correlated, our study clearly demonstrated that the rate of apoptosis was more severe in the cortex than in the medulla. Furthermore, in the adrenal cortex the distribution of apoptosis was heterogeneous. It was more prominent in *zona reticularis*, followed by the most inner half of *zona fasciculata* and then decreased progressively towards *zona glomerulosa*, where it was practically absent.

The different anatomical distribution of apoptosis in the adrenal gland of rats has been described previously. In fact, in this specie each zone of the adrenal gland behaves independently in terms of apoptosis and cellular proliferation (Bozzo et al., 2006). Moreover, experimental studies in rats have shown that both dexamethasone administration and hypophysectomy (which leads to decreased ACTH levels) increased apoptosis mainly in *zona reticularis* (Carsia et al., 1996; Almeida et al., 2007). In Humans too, the rate of apoptosis was found to be higher in the cortex than in the medulla (Wolkersdörfer et al., 1996).

The reasons for the heterogeneous distribution of apoptosis were not determined in this study. Nevertheless, based in the literature, several hypotheses can be advanced. In part it may reflect the occurrence of paracrine and/or autocrine regulatory mechanisms which are specific to each anatomical zone (Bozzo et al., 2006). A heterogeneous anatomical distribution of proteins involved in the apoptotic process has also been demonstrated and this can also be a contributing factor. For example the concentration of superoxide dismutase is different accordingly to the, different zones of the adrenal cortex (Vinson 2016).Three other examples include the IL6 receptor which is mainly found in *zona reticularis* and inner *zona fasciculata* (Päth et al., 1997), thrombospondin-2, a trimeric matricellular protein with anti-apoptotic properties, which is absent from *zona reticularis* (Feige, Keramidas & Chambaz,

1998) and Bak, a well-known pro-apoptotic protein, which is only found in the cortex (Krajewski, Krajewska & Reed, 1996). In addition,factors related with adrenal vasculature may also influence the different susceptibility to apoptosis. As described previously, the inner zones of the adrenal cortex are more prone to suffer from ischemia (Sparrow& Coupland, 1987), especially hypotensive states such as HS (Jasper et al., 1990; Hamaji et al., 1986). Besides, the capillary walls of *zona reticularis and fasciculata* have larger fenestrations than those of *zona glomerulosa* (Motta, Muto & Fujita, 1979), which at least theoretically can facilitate the diffusion of pro-apoptotic substances present in plasma into the adrenal interstitium. Finally, it is also known that macrophage distribution in the adrenal gland is heterogeneous (Schober, Huber, Fey & Unsicker, 1998), with these cells being mostly predominant in the inner cortical regions (Almeida, Ferreira & Neves, 2004), in close proximity to capillary wall fenestrations (Motta et al., 1979). Because macrophages have a pivotal role in the development of adrenal inflammation (Wang et al., 2015; Liu, Tipoe & Fung, 2014) and the latter is related with apoptosis, this particular distribution can contribute to the higher occurrence of apoptosis in the inner parts of the adrenal cortex.

Was apoptosis associated to adrenal dysfunction in our model?

The higher rate of apoptosis found in the inner parts of *zona fasciculata* and *zona reticularis* at least theoretically could be associated to a decrease in glucocorticoids and androgens levels, accordingly to the functional zonation of the adrenal cortex (Vinson, 2016). Thus the hypothesis that adrenal apoptosis was associated to decreased CS levels in G2 (and G3, described later) seems plausible. However, we were not able to find a significant correlation between the level of apoptosis and the levels of CS, which contrasts with what has been found by others (Yu et al., 2016, Kanczkowski et al., 2013a; Kanczkowski et al., 2013c; Yu et al., 2012).

This difference can be explained, at least partially, by several reasons. In two of the studies, both in ANP, the maximal decrease in CS levels occurred at 24 hours after induction of ANP, when the apoptotic index reached a value of approximately 12%-25% (Yu et al., 2016; Yu et al., 2012). In our study the maximum apoptotic index was observed at 4 hours after induction of HS and was considerably lower (approximately 8%). These results suggest that in order that apoptosis is associated to significant adrenal dysfunction, a minimal number of cells must be affected. Because the latter may be time-dependent, it is possible that if our experiment lasted longer, apoptosis would affect a larger number of cells. Maybe in these circunstances CIRCI would become more manifest. Another reason that can explain the lower apoptotic rate of our study is the mild degree of hypotension, because in HS it was demonstrated thatthe degree of tissue apoptosis is related with the duration and magnitude

of hypotension (Moran et al., 2008). Consequently it is possible that a more severe HS model result in a higher degree of adrenal apoptosis and maybe a more clear evidence of apoptosis-associated adrenal dysfunction. Alternatively the nature of the underlying pathology can also influence the rate of adrenal apoptosis. In fact it must be considered that ANP, septic shock and HS are very different pathological conditions and can have a different influence in the development of adrenal apoptosis.

Although there is some debate, as described previously, the majority of authors consider the inner portion of the rat adrenal cortex as *zone reticularis*, and this a the area of synthesis of both glucocorticosteroids and androgens (Vinson, 2016). Consequently, it might be speculated that increased apoptosis in *zona reticularis* can lead to a significant decrease in adrenal androgens levels. This could provide one of explanations for the decrease of DHEAS levels commonly found in critically ill patients (Arlt et al., 2006; Beishuizen et al, 2002).

5.3. Analysis of G3

5.3.1. Hormonal variables

Etomidate-treated animals had impaired CS secretion when compared with the other three groups. This was an expected finding due to the known effects of etomidate in adrenal function (Cotten et al., 2009; Preziosi and Vacca, 1982). However, the magnitude of the decrease was not expected. The levels of CS which were achieved in G3 were lower than those reported in normal rats after etomidate administration (Cotten et al., 2009). At several time points they were even lower than those reported in G1 and at T0 lower than in G0. They were also below the level of 100000 pg/ml at T2. Our results showed that the adrenal response to HS was completely blunted by etomidate, despite the stimulus provided by the high levels of ACTH. The pattern of ACTH secretion was also different to the one which was observed at G2.

To our knowledge there are no studies in rats which have described the influence of etomidate in the adrenal response to HS. However, there is literature that describes its effects in the stress response to several stimuli (including HS) in other species. In dogs the administration of etomidate did not influenced the stress response to induced hypoxia (Hirschman et al., 1991) or elective surgery (Dodam, Kruse-Elliot, Aucoin & Swanson, 1990). However in HS, etomidate decreased plasma levels of progesterone, 17 alpha-hydroxyprogesterone, CS, cortisol, and aldosterone (Fraser et al., 1984). The decrease in these hormones occurred despite amassive rise in ACTH, renin, and angiotensin II levels following HS. In Humans, etomidate also effectively suppressed the adrenal response to surgery (Forman, 2011; Mehta et al., 1985).

The influence of etomidate in the adrenal response was mostly studied in sepsis, especially in the experimental setting. In murine models of septic shock, including CLP and LPS administration, etomidate was able to diminish the adrenal response to sepsis, but did not completely abolished it (Liu et al. 2016; Santer et al., 2015; Liu et al., 2015; Pejo et al., 2012). Moreover, in these studies etomidate had no influence in the normal ACTH response. In another study of sepsis, this time in anesthetized pigs, metomidate, an analogue of etomidate was used to suppress cortisol secretion. It was found that the resulting cortisol deficiency facilitated the development of circulatory failure secondary to *Pseudomonas* spp bacteremia (Neumann et al., 1989).

The results of these studies differed significantly from our model, especially in the magnitude of glucocorticoid suppression and in terms of ACTH secretion. These differences could in part be related with differences in experimental methodology. First, some of the studies were performed in other species than rats, especially dogs and pigs and it is known that some aspects of the stress response are specie specific (Herman et al., 2016). In addition, the nature of the stressful stimulus was different, with most studies being concerned with sepsis. The nature of the stressful stimulus can influence the characteristics of the stress response. For instances it is known that in rats the administration of LPS leads to a greater elevation of ACTH and CS than HS (Molina & Abumrad, 2000). Likewise it was also demonstrated in rats that HS and LPS cause systemic inflammation by different mechanisms (van Wessem et al., 2013).

The dosages of etomidate which were used were also different between studies, which can be itself a source of variation. Besides the animals of our study were anesthetized, which contrasts with one of the cited studies, where the experiments were conducted in conscious animals (Pejo et al., 2012). This is also relevant, because general anesthesia can affect the HPA response to stressors, especially by decreasing ACTH secretion (Leal & Moreira, 1996). Finally, we cannot rule out that the pharmacokinetic and pharmacodynamics of etomidate were differently influenced by experimental conditions (e.g. HS *vs* septic shock). A suggestion that this might have happened was that in our study, the duration of adrenal suppression, estimated by the decrease in CS levels, lasted around 240 minutes, whereas in the Pejo et al., (2012) study it lasted 60-120 minutes (Pejo et al., 2012). The possible role of HS in influencing etomidate´s pharmacokinetic and pharmacodynamics will be discussed in the next sections.

Why were the levels of CS so low in our model?

There are two possibilities that can justify the low levels of CS in G3. One is that CS synthesis and secretion were decreased and the other is that CS metabolism was increased. We believe that the main reason was decreased synthesis of CS. In turn we hypothesize that this was mainly due to etomidate-associated adrenal suppression. In addition, decreased CS synthesis might have resulted from adrenal dysfunction, which similar to G2, could have been related with apoptosis, necrosis and inflammation.

G3 animals had also higher rates of adrenal gland apoptosis and necrosis than G1 and G0. However, they were lower in G3 than in G2. If apoptosis and necrosis were the main cause for the decreased CS levels observed in etomidate-treated animals, than G2 would have significantly lower CS levels. Because the opposite occurred, we believe that in G3 the contribution of apoptosis/necrosis to decreased CS synthesis, if present was more limited than in G2. Apoptosis and necrosis will be discussed in more detail in later sections.

As stated in previous sections, adrenal dysfunction could develop following increased inflammation by two mechanisms: direct effects of systemic cytokines and development of intra-adrenal inflammation. Etomidate-treated animals had higher cytokine levels (discussed later), including TNF-α and IL6. Because these are known to stimulate steroidogenesis it is unlikely that its increase contributed to CS low levels. By contrary, we cannot completely rule out the role of IL10 in this regard, because this cytokine decreases CS production (Elenkov, 2004).

If etomidate-treated animals had high intra-adrenal inflammation is unknown as this was not evaluated in this study. By one side, increased systemic inflammation would favor the development of intra-adrenal inflammation. On the other side, a recent study found that etomidate administration was associated to decrease systemic TNF- α and IL6 levels and decreased intra-adrenal inflammation, through decreased TLR-4 expression and inhibition of NF-kB nuclear translocation (Zhang et al., 2015). Because intra-adrenal inflammation was not assessed in this model, its contribution to current findings remains unclear.

Regarding the possibility that increased CS metabolism was responsible for the decreased CS levels, we think that this hypothesis is highly unlikely. In Humans cortisol´s half-life is estimated to be around 60-120 minutes. Cortisol´s depuration is mostly carried out by the liver, through A-ring reductases (5β-reductase and 5α-reductase) and by the kidney, through 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which converts cortisol to cortisone. Cortisone is than excreted on the urine (Boonen et al., 2013). In rats, 11β-HSD also catabolizes the conversion of CS into 11-dehydrocorticosterone, which is its inactive form (Wang et al., 1999; Musajo et al., 1996). It is known that etomidate does not increase 11β-HSD activity (Quinkler et al., 2007). In addition, current evidence suggests that corticosteroid

clearance is decreased during critical illness due to reduced activity of the correspondent metabolizing enzymes patients (Boonen et al., 2013). This has also been demonstrated in rats submitted to HS (Wang et al., 1999).

The suppression of adrenal production of CS by etomidate

In this study, etomidate´s effects in adrenal suppression seemed to be potentiated. The most likely cause for this was that HS induced changes in etomidate´s pharmacokinetics and pharmacodynamics. In fact, it has been demonstrated that HS decreases etomidate´s clearance and distribution volume. Thesecan potentially increase the drug´s duration of action and potency (Johnson et al., 2003; de Paepe et al., 1999). This issue will be more extensively discussed in the section of hemodynamic variables.

Was etomidate associated to CIRCI´s in this model?

As described before CIRCI is defined as an inadequate glucocorticoid anti-inflammatory activity in critically ill patients due to either insufficient glucocorticoid availability or due to the presence of resistance/insensitivity to glucocorticoid actions at the cellular level (Marik, 2009). Because the physiological response to stress is an increase in corticosteroid levels, one approach to diagnose CIRCI in critically ill patients is to measure total random glucocorticoid levels. These should be measured without exogenous stimulation and in the presence of stressful stimulus (e.g. hypotension), which are known to strongly stimulate glucocorticoid production. Although theoretically sound, there are some problems with this approach. Total plasma cortisol concentration is the result of several variables including its production and secretion at the adrenal, distribution, binding and elimination (Gibbison et al., 2013). In addition cortisol is secreted in a pulsatile manner and has circadian variation (although the latter may be lost during critical illnesses). Furthermore, total plasma concentrations give little information about glucocorticoid function. Only free cortisol can cross cell membranes. Only after it can and exert its function by binding to the glucocorticoid receptor and after the subsequent complex moves into the nucleus (Boonen & Van den Berghe, 2016). Lastly, which levels of CS are considered adequate for critical illness is still unknown. The current understanding is that under stress situations, “low” baseline glucocorticoid levels have a high positive likelihood ratio to diagnose CIRCI, and “high” baseline cortisol has high negative likelihood ratio to diagnose CIRCI. Most experts agree that a value of cortisol of 10 µg/dL is low, and that baseline cortisol greater than 34-44 µg/dL is high.

In 2008, the American College of Critical Care Medicine developed a consensus statement with recommendations for the diagnosis and management of corticosteroid insufficiency in critically ill patients (Marik et al., 2008). This consensus statement proposed that a baseline cortisol <10 µg/dL or cortisol variation after 250 µg of cortrosyn < 9 µg/dL were diagnostic criteria for CIRCI. This recommendation was mainly based in the study of Annane et al., (2006), which found that a baseline cortisol <10 µg/dL had a high specificity in CIRCI diagnosis (specificity, 1; 95% CI, 1-1), although it had a low sensibility when compared with the gold standard (the metyrapone test) (Annane et al., 2006). Nevertheless, some experts showed the limitations of this recommendation (Moraes, Czepielewski & Friedman, 2009) and in the last Surviving Sepsis Campaign (Dellinger et al., 2012) the use of the ACTH test to diagnose CIRCI was discouraged. Instead, the guidelines suggest that a random cortisol level <18 µg/dL in patients with septic shock and/or clinical suspicion of adrenal failure were indications for initiating steroid therapy. Although these recommendations aimed to facilitate the diagnosis of CIRCI, they have the disadvantage of being largely based in expert opinion.

Other authors have suggested the use of a mixed approach to the diagnosis of CIRCI (Moraes et al., 2009). In this approach, adrenal dysfunction is diagnosed when baseline cortisol is inadequately low (< 10 µg/dL), and excluded when baseline cortisol is > 34-44 µg/dL. In patients with baseline cortisol between 10 µg/dL and 34 µg/dL, the authors suggested to use the ACTH stimulation test to rule out adrenal dysfunction (Moraes et al., 2009).

All this considered, despite its limitations, the measurement of total random glucocorticoid levels in the presence of stressful stimulus, is still considered one of the current and reliable methods of diagnosing CIRCI. In addition the determination of basal cortisolemia was the test used to diagnose CIRCI in conditions where etomidate has been administered (Asehoune et al., 2012). There is even literature that suggests that levels of total random cortisol □10 µg/dL and □25 µg/dL are indicative of severe and relative hyperacute adrenal insufficiency, respectively in Humans with HS (Stein et al., 2013). It was based in this literature that in our study, we considered that levels of CS below 10 µg/dL were diagnostic of CIRCI. Thus, based in this assumption, in the published definition of CIRCI (Marik, 2009) and in the fact that CS levels were lower than 10 µg/dL (10000 $pg.ml^{-1}$) (Marik et al., 2008) we considered that animals of G3 had CIRCI. In support of our assumption we also have the results of other experimental studies, where rats with ANP and HS considered to have CIRCI had CS levels of similar magnitude to our results (Yu et al., 2016; Yu et al., 2012; Rushing et al., 2006; Wang et al., 1999).

To our knowledge this is the first experimental study in HS that associated etomidate administration to development of CIRCI. As it will be shown in the following sections, we believe that etomidate-associated CIRCI was associated to significant morbidity.

ACTH secretion in etomidate-treated animals

In etomidate-treated animals the pattern of ACTH secretion was different from the pattern found in G2. In fact, despite the progressive decrease of CS levels from T1 to T3, a compensatory increase in ACTH secretion did not occur. The levels of CS and ACTH seemed dissociated and the normal feedback mechanism of the HPA axis appeared to be impaired at acentral level. In addition ACTH levels continuously decreased from T1 to T3.

Even knowing that in rats, the stimulation of the HPA axis occurs mainly by corticosteroid-independent mechanisms (Thrivikraman & Plotsky, 1993), our findings seem odd, because etomidate–treated rats had also other conditions which are known to stimulate the release of ACTH, including increased TNF-α and IL6 levels (although these can also decrease ACTH secretion-see below), worsening hypotension and hypoxemia.

Thus, it seems that somehow the secretion of ACTH was impaired, although the reason for that was not determined. Most likely this was not the result ofa direct effect of etomidate because this drug is associated to increased ACTH secretion (Preziosi and Vacca, 1982). The same goes for a possible interaction of etomidate with isoflurane, because isoflurane does not affect the stress response in rats (Wu et al., 2015).

In septic and post-surgical Human patients, a decrease in the levels of ACTH has been reported (Annane 2016; Cho et al., 2000). Similarly, experimental studies in rats have also showed decreased ACTH secretion in the later stages of severe HS (Annane 2016; Grässler et al., 1990). The same has been identified in rat models of sepsis, where ACTH secretion was not only decreased but also dissociatedfrom the levels of CS (Zhang et al., 2015). Some authors argue that the decrease in ACTH may be an adaptive mechanism to critical illness with the aim of decreasing catabolism (Vogeser, Zachoval, Felbinger & Jacob, 2002). Nevertheless, the mechanisms behind this response are still incompletely understood. One of the proposed mechanisms is increased cytokine levels such as TNF-α, IL1 and IL6. In fact, the stimulation of the hypothalamic/pituitary axis by cytokines is characterized by a biphasic response. Initially, cytokines such as IL1 and TNF-α increase ACTH secretion. This is followed by a progressive decline in anterior pituitary ACTH concentrations (Parsadaniantz et al., 1997; Mélik et al., 1994). The biphasic response to cytokines seems to be due to increased local levels of NO (Annane, 2016; McCann et al., 2000). Increased expression of inducible NO synthetase has been demonstrated in hypothalamic parvocellular neurons during states of systemic inflammation, including sepsis (Polito et al., 2011, Wong et al., 1996). Other mediators which have been implicated in the decrease of ACTH synthesis include ANP, endothelin, macrophage-inhibitory factor (MIF) and cortisol itself (Beishuizen et al., 2001; Cho et al., 2000; Wang et al., 1999; Vermes et al*.,* 1995). Although not proven, and based on these studies, it is possible that the increase in cytokine levels of etomidate-treated

animals was associated to the late decrease in ACTH synthesis, through a NO-mediated mechanism. The role of other mediators such as endothelin, ANP or MIF is uncertain because they were not evaluated.

5.3.2. Immunological variables

To our knowledge this is the first study which described the impact of etomidate administration in the cytokine response to experimental HS. Curiously, etomidate-treated animals had significantly higher levels of IL6 and IL10 than G2 and were the only group with significant increases in TNF-α levels above the limits of detection. This suggests that etomidate administration was able to significantly augment the normal cytokine response to HS.

The use of etomidate has been associated to increased cytokine levels in other settings, including rat models of septic shock (Santer et al., 2015; Pejo et al., 2012) and in women submitted to hysterectomies (Jameson et al*.,* 1997). However there is also evidence that suggests the contrary. In Human patients submitted to cardiac bypass, Fillinger et al., (2002) found that patients which had etomidate administration for endotracheal intubation had lower IL6 and IL10 levels. Identical results were obtained by Zhang et al., (2015) in a CLP model (Zhang et al., 2015). In contrast, in obese patients submitted to gastroplasty, etomidate decreased cortisol levels but did not influence post-operative levels of TNF-α when compared with controls (Montalba et al., 2001). Why etomidate administration led to the different results of these studies is poorly understood. Possible explanations include species differences, nature of the stressful stimulus, concomitant influence of other anesthetics and/or medical interventions and different doses of etomidate. The latter seemed particularly important because etomidate´s adrenal suppression of glucocorticoid production is dose-dependent (Cotten et al.¸2010). As it will be discussed in the following sections, CS levels have a major impact in how they influence the immune system.

We believe that the increased cytokine levels observed in etomidate-treated animals may have been caused by several possibilities:

- Decreased glucocorticoid levels, caused by etomidate´s adrenal suppressive effects;
- Direct immune effects by etomidate
- Due to etomidate-associated increase in tissue oxygen debt and hypoperfusion

These possibilities will be discussed in the following sections.

Did etomidate increase cytokine levels through its effects in glucocorticoid production?

There are several lines of evidence that suggest that the increased cytokine levels were due to the decreased CS levels caused by etomidate.

The first line of evidence is that glucocorticoids are well known to supress cytokine production. This inhibition may result from occlusion of positive responsive sequences by binding of the glucocorticoid receptor to specific DNA domains, by direct interaction of glucocorticoid receptor with subunits of the transcription factor NF-kB, or through the decrease of IL6 mRNA stability. The inhibition of cytokine production by glucocorticoids has been demonstrated in several experiments. Cytokines decreased by glucocorticoids include TNF-α (Bertini et al., 1988), IL1 (Staruch & Wood, 1985) and IL6 (Amano et al., 1993; Morrow et al., 1993). It has also been demonstrated that hydrocortisone infusion reduces plasma levels of IL6 in patients with septic shock (Oppert et al., 2005).

The second line of evidence is based in several studies which have demonstrated the role of glucocorticoids in modulating the cytokine response to HS. Basal cortisol concentrations were found to be inversely correlated with the increase in IL6 concentrations following experimental haemorrhage–reinfusion (Komaki et al., 1994). Also adrenalectomised rats submitted to HS have increased levels of IL6 (Komaki et al., 1994), TNF-α (Yamashita *&* Yamashita, 2001) and tissue mRNA expression of several cytokines including IL1α, IL1β, IL1ra, IL6 and TNF-α (Goujon et al., 1996), when compared to control rats with intact adrenals. Similarly, both surgical and pharmacologic adrenalectomy with mifepristone increases IL6 levels in rats (Goujon et al., 1996; Morrow et al., 1993).

It may be argued that increased cytokine levels observed in adrenalectomised rats result not only from the lack of glucocorticoids but also from decreased catecholamine levels. This is because in conditions such HS, increased catecholamine levels have a suppressive effect in pro-inflammatory cytokines (Molina & Abumrad, 2000; Szabó et al., 1997). Nevertheless current evidence suggests that in the adrenalectomised animal model it is the lack of glucocorticoids which has the most significant effects on cytokine levels. For instances, the stimulating effects of propranolol in TNF-α production by peritoneal macrophages are enhanced in animals lacking circulating levels of glucocorticoids (Stanojevic et al., 2013). In addition, adrenalectomised rats supplemented with exogenous glucocorticoids achieve CS levels to those of normal rats with HS have TNF-α levels similar to controls with HS and without adrenalectomy (Yamashita & Yamashita, 2001). In contrast the same results were not obtained when adrenalectomised animals were supplemented with epinephrine (Yamashita & Yamashita, 2001).

The third line of evidence is based in clinical studies which have demonstrated that CIRCI is associated to increased levels of TNF-α, IL6 and IL10. This has been demonstrated in

surgical, trauma, brain injury and septic patients (Kwon et al., 2010; Kashiwabara et al., 2007; Dimopoulou et al., 2004; Hoen et al., 2002).

Finally the fourth line of evidence is based in the fact that decreased production of glucocorticoids by etomidate has been associated to increased cytokine levels in several studies (den Brinker et al., 2005; Yeager et al., 2005; Jameson et al., 1997).

Based on this evidence, we believe that major contributor for the increased cytokine levels in G3 animals was the suppression of adrenal production of CS by etomidate. In this line of reason, without the restraining effects of endogenous CS, the normal cytokine response to HS was magnified. Our results also showed that animals of G3 had already increased cytokines at T0 when compared with other groups. Surprisingly, the low levels of CS at T0 could have also played a secondary role in the subsequent increase in cytokine levels. The relationship between endogenous glucocorticoids with the immune system is more complex than once thought. Accordingly to several authors, the role of glucocorticoids in the endogenous stress response includes both stimulatory and suppressive effects, depending from its dose and time of release (Sapolsky, Romero & Munck, 2000; Munck & Naray-Fejes-Toth, 1994; Munck, Guyre & Holbrook, 1984). Therefore, when the body is threatened by a stressor, in the initial stress response, glucocorticoids have “a permissive action in the immune system” by allowing and even enhancing the inflammatory response to injury. This includes an enhancing effect in cytokine synthesis and release (Sapolsky, Romero & Munck, 2000; Lim, Müller, Herold, van den Brandt & Reichardt, 2007). The action of glucocorticoids does seem to describe a bell-shaped curve. In this model glucocorticoid stimulatory effects in cytokine actions are observed when they are at low levels and immunosuppressive effects when they are at higher levels (Sapolsky, Romero & Munck, 2000; Munck & Naray-Fejes-Toth, 1994; Munck, Guyre &Holbrook, 1984). Thus, it is possible that at T0, the low levels of CS permitted an enhancement of cytokine secretion. Only later, after HS has developed, the low levels of glucocorticoids, combined with other factors (e.g. release of DAMPs, I/R injury) were associated to an overactivation of the immune system. The model proposed by Sapolsky, Romero & Munck, (2000) can also justify the findings of Komaki et al., 1994. In this study, adrenalectomised rats submitted to HS and with plasma CS maintained at normal or slightly elevated levels through supplementation had higher basal plasma levels of IL6 and a higher increase in IL6 after HS than sham-operated rats (Komaki et al., 1994). Accordingly to the theory suggested by Sapolsky, Romero & Munck, (2000), adrenalectomised rats which did not had supplementation could not mount a cytokine response to HS because they lack the permissive action of CS. Cytokine production was then completely restored with CS supplementation. These studies clearly show that the degree and time of adrenal suppression may influence the correspondent cytokine levels.

Not all increased cytokine activity was related to decreased CS levels however. The best example, are the increased levels of IL10. Although endogenous glucocorticoids are known to inhibit IL10 secretion and production (Brattsand & Linden, 1996), IL10 levels reached their peak when CS levels were lower than 10 µg/ml.

A possible explanation for the increased IL10 levels was that this cytokine was triggered by increased TNF-α and IL6 levels. In fact, TNF-α and IL6 are known to induce IL10 secretion and production (Brøchner & Toft, 2009). Corroborating this hypothesis we found a positive correlation between TNF-α, IL6 and IL10. Another possibility could have been the release of DAMPs during ischemia/hypoxia or I/R injury because this stimulates IL10 secretion (Maslanik et al., 2013).

Did etomidate increase cytokine levels through its direct immune effects?

Although less likely, a direct effect of etomidate in increasing cytokine production is also possible. Several studies have shown that etomidate has direct immune effects. It is known that etomidate decreased the expression density of CD14 in blood monocytes and increased IL1ra and IL10 release by cultured human whole blood stimulated with endotoxin (Larsen et al., 1998). Etomidate also interfered with circulating lymphocyte levels (Cherfan et al., 2011) and decreased neutrophil chemoluminescence, an index of ROS generation, essential for neutrophil bacterial killing capacity (Gelb & Lok, 1987). Etomidate also decreased the release of pro-inflammatory cytokines and CD14 expression in rat macrophages exposed to LPS (Liu et al., 2015). A recent study showed that etomidate in a lower dose than the one we used (0.6 mg/kg IV, followed by infusion) was able to decrease TNF-α and IL6 (Zhang et al., 2015). How etomidate exerted these effects is not completely clarified although it was shown that etomidate decreased TL4 expression and inhibited NFκB translocation (Zhang et al., 2015).

Thus available evidence supports that etomidate has direct immune effects. However, because they are mostly anti-inflammatory it is highly unlikely that they were behind our findings except probably the increased IL10 levels.

Did etomidate increase cytokine levels by increasing tissue hypoperfusion and tissue oxygen debt?

Our findings support the hypothesis that etomidate might have increased cytokine levels indirectly by increasing tissue hypoperfusion and tissue oxygen debt (this subject will be discussed later). In fact, several studies have demonstrated that the severity of the inflammatory response to HS is proportional to the degree of oxygen debt (Sato et al., 2010; Bitterman et al., 1991).Corroborating this, in etomidate-treated animals a negative correlation

was found between the levels of TNF-α, IL6 and IL10 and pH, BE and HCO_3. Thus the degree of metabolic acidosis (manifested by a decrease in pH, BE and HCO_3) was negatively correlated with the higher cytokine levels.

We hypothesize that by increasing tissue hypoperfusion and oxygen debt, etomidate led to increased tissue injury. This in turn, led to increased release of DAMP´s and activation of tissue immune cells such as macrophages. The result was an increased cytokine production *in loco*, which was also facilitated by the inadequate levels of systemic glucocorticoids.

Actually, the highest levels of TNF-α, IL6 and IL10 were observed at T2 and T3, after resuscitation. This finding could have several explanations. The first is that it was due to volume expansion associated with resuscitation. This caused a washout effect of locally produced cytokines, which then gained access to the systemic circulation (Nedrebo et al., 2004). Alternatively this increase could represent a true increase in cytokine secretion due to I/R injury, associated to resuscitation (Bennetts et al., 2014, Nedrebo et al., 2004).

It might also be hypothesized that etomidate increased cytokine levels by potentiating I/R injury because studies have shown that the anesthetic has pro-oxidant effects (Liu et al., 2015; Wang et al, 2014; Yagmurdur et al., 2004). To our knowledge no studies have evaluated the effects of etomidate in I/R injury following HS. However in other conditions, the majority of studies have shown a protective effect by etomidate in this regard, although this may be organ-specific. Etomidate attenuated reperfusion injury in the skeletal muscle (Ergün et al., 2010) and spinal cord (Yu et al., 2010), but not in the kidney (Yuzer et al., 2009). Thus, and although a role of etomidate in the modulation of /I/R injury cannot be ruled out completely, we believe that if this occurred, was most likely protective. Consequently, it most likely did not contribute to theincreased cytokine levels.

5.3.3. Influence of etomidate on metabolic and hemodynamic variables

Etomidate-treated rats had higher levels of lactate and lower levels of pH, HCO_3 and BE. Etomidate-treated rats had also lower levels of PO_2.Increased lactate and decreased pH, HCO_3 and BE levels are used to indicate decreased tissue perfusion and higher tissue oxygen debt in HS (Rixen & Siegel, 2005). This indicates that etomidate-treated animals had worse tissue perfusion and higher tissue oxygen debt than animals of G2.

We hypothesize that several reasons could have justified why etomidate-treated animals had worse tissue perfusion and higher tissue oxygen, and these are:

- Higher degree of arterial hypoxemia
- Interference of etomidate in the macrocirculation
- Interference of etomidate in the microcirculation

Importantly we hypothesized that these factors worked and interacted in combination to induce and increase oxygen debt.

Hypoxemia and etomidate

The higher tissue oxygen debt of etomidate-treated animals could have partially resulted from their higher levels of arterial hypoxemia. This in turn could have been due to two major mechanisms. The first is the known effect of etomidate in increasing the alveolus-arterial oxygen gradient and intrapulmonary shunting (Criado, Maseda, Garcia Carmona, Dominguez & Avello, 1983). The second was the development of progressive lung dysfunction, manifested by a gradual decrease in PO_2/FiO_2 ratio, which was lower in G3 than in G2. Eventually the PO_2/FiO_2 ratio reached levels lower than 300, which is considered the cut-off value to diagnose acute lung injury (ALI) in Human patients (Costa & Amato, 2013). Based in the PO_2/FiO_2 ratio, animals of G3 not only had severer lung dysfunction, but also had evidence of ALI at T2 or T3. This raises the question on how etomidate was associated to increased lung injury.

Did etomidate cause ALI?

Theoretically both HS groups were exposed to the same risk factors that predispose to ALI development. Two of these risk factors are HS itself (Sato et al., 2010) and mechanical ventilation (van Wessem et al., 2013). The risk is magnified if both factors are present in the same patient, because they interact together to increase lung and systemic cytokine release (Bouadma et al., 2007). Another risk factor which can be considered was lung exposure to 100% oxygen during several hours. 100% oxygen supplementation is commonly performed in HS models, because it reduces tissue dysoxia and prolongs survival (Takasu et al., 2009). However, lung exposure to 100% oxygen for extended periods can lead to hyperoxic acute lung-injury (HALI) (Yu et al., 2015; Kallet & Matthay, 2013). HALI constitutes a subtype of lung injury which results from increased production of ROS. Its severity depends from several factors including duration of exposure, FiO_2 (especially if FiO_2 is higher than 0.6), genetic and specie-related factors and presence of underlying lung pathology (Kallet & Matthay, 2013). Therefore it is possible that the animals of our study suffered some degree of HALI. Nevertheless, if occurred it was most likely limited in severity, as hyperoxia induced-lung damage is especially apparent at 12 hours of exposure to 100% oxygen (Yu et al., 2015) and our animals had a maximum exposure of 8 hours.

Resuscitation in both HS groups consisted in a large volume of crystalloids and blood. This resuscitation regime has been associated with increased survival in HS models (Frink et al.,

2011; Takasu et al., 2010). However, resuscitation with large volume of crystalloids has also been associated to increased lung inflammation and injury in rats submitted to HS (Yu et al., 2014) and increased incidence of pulmonary complications in Humans with clinical HS (Cotton et al., 2006). Besides, it is now recognized that fluids used in resuscitation of HS are not innocuous. Depending of the type of fluid they can increase cytokine production (Lee et al., 2005) and potentiate cellular injury (Alam, 2006). The use of blood products can be particularly deleterious, because its use has been associated to early immune activation resulting in SIRS and MOF (Siliman, Moore, Johnson, Gonzalez & Biffl, 2004). Consequently we cannot rule out that our resuscitation regime also contributed to development of lung injury in HS groups.

Nevertheless, only etomidate-treated animals had lung injury which was severe enough to be considered ALI, which argues for a causative role of etomidate in this condition. Interestingly etomidate has been previously linked to development of lung complications. It was associated to increased risk of developing ARDS (Warner et al., 2009) and hospital-acquired pneumonia (HAP) in trauma patients (Asehnoune et al., 2012). In addition in a study of pigs submitted to experimental bacteremia with *Pseudomonas aeruginosa*, the animals which had metomidate, an analogous of etomidate developed pulmonary edema (Neumann et al., 1989).

Etomidate might have contributed directly to ALI development, through its direct immune effects (see before) or through its pro-oxidant activity (Wang et al., 2014; Yagmurdur et al., 2004) and ability to increase neutrophil oxidative burst activity (Szekely, Heindli, Zahler, Conzen & Becker, 2000). Etomidate´s pro-oxidant activity would be particularly deleterious in HS, where increased ROS production is associated to increased tissue injury and microvascular permeability, including the lung (Bennetts et al., 2014). To our knowledge, no studies are available to support a direct role of etomidate in inducing lung injury.

Alternatively, etomidate could have contributed to lung injury indirectly, by suppressing endogenous CS production. In this line of reasoning, the relative lack of endogenous CS potentiated the lung inflammatory response to HS, mechanical ventilation, HALI and resuscitation. Increased systemic inflammation caused also by decreased CS levels could have also contributed to lung injury as well. There is clinical evidence that supports this hypothesis. In the study where etomidate was associated to the development of HAP in trauma patients, HAP was partially prevented by hydrocortisone administration (Asehnoune et al., 2012). The authors suggested that etomidate exposed patients to HAP, at least partially, by inducing an excessive inflammatory response. They affirmed that a low-dose of hydrocortisone was beneficial by offsetting adrenal suppression and having immune enhancing effects. Furthermore, the administration of glucocorticoids was shown to prevent

the development of ALI in surgical patients (Park, Lee, Jang, Joo & Zo, 2012) and to decrease morbidity and mortality in ARDS (Tang, Craig, Eslick, Seppelt & McLean, 2009).

Interference of etomidate in the macrocirculation

In this study etomidate was associated to significant changes in HR and MAP, when compared with the other groups. Etomidate-treated animals had lower HR in most time points (and comparing with G2, at all time points). They also had lower MAP from T0 to T1. We believe that these cardiovascular effects could have played a role in increasing hypoperfusion and tissue oxygen debt in etomidate-treated animals.

At a first look our findings seem to contrast with what has been described in the literature. Etomidate is often chosen for anesthetic induction in critically ill patients because it better preserves hemodynamic stability (Forman, 2011). For the same reasons it is considered the ideal choice for providing rapid sequence intubation in critically ill patients by many emergency clinicians (Forman, 2011). The concept that etomidate has beneficial cardiovascular effects was in part derived from experimental studies, including in several models of HS. In 1985 Peterson & Hoffman, showed that the administration of etomidate to rats with HS was not associated to increased mortality (Peterson & Hoffman, 1985). In a study with dogs submitted to hypovolemia Pascoe et al., (1992) reported that etomidate administered as an IV bolus was associated to minimal cardiovascular changes (Pascoe et al., 1992). Johnson et al., (2003) also demonstrated that etomidate was associated to minimal cardiovascular changes in a swine model of HS. In another study but in this case in dogs submitted to HS which subsequently received etomidate or ketamine, etomidate was found to preserve hemodynamic stability (Fraga et al., 2006).

Based in this studies etomidate seemed indeed a good candidate for anesthetic induction in critically ill patients and especially when there are concerns about hemodynamic stability. However, these studies did not demonstrated that etomidate was deprived of side-effects and consequently, that the use ofetomidate in HS was completely safe. By contrary a careful and objective analysis of the literature suggests that a more prudent approach should be adopted regarding the safety of etomidate in HS.

First there is the concern that because many of the existing studies were performed in different species (dogs, rats, swine) the fact that etomidate seems safe in one specie does not mean that the same is true in otherThis is because specie-related differences may exist. For instances. the doses of etomidate which are required to promote adequate sleep duration and hypnosis are higher in rats (2-5 mg/kg) than in humans (0.3 mg/kg) or dogs (0.8-2.5 mg/kg). In addition, in rats etomidate is associated to a higher incidence of cardiovascular side effects than in other species (Wildt et al., 1983). Furthermore, one

should note that , even when the studies regarding etomidate´s safety were performed in the same species the dosage and route of administration were different between studies and these factors may influence the development of side-effects. The same reasoning can be applied to the fact that the time point in relation to HS of etomidate administration varied between studies (e.g. before vs after HS), Otherexperimental conditions also varied between studies, including the use and/or type of fluid resuscitation, mechanical ventilation and other the other anesthetics or analgesics which were added to etomidate. In fact a closer and critical look into the studies reveals that etomidate induced several significant side-effects including hypotension and cardiac depression (Wildt et al., 1983).

After these considerations, it becomes clear that our results do not conflict with what has been published in the literature.In fact they actually, illustrate that etomidate is not deprived of side-effects and that these can be deleterious in specific circumstances. We believed that this is what occurred in our experiment.

The lower HR found in etomidate-treated animals was most probably unrelated with etomidate itself because the drug, even when at high concentrations, is not associated to a negative chronotropic effect (Zausig et al., 2009). We hypothesize that the lower HR was due to the metabolic acidosis. In fact in a rat model of HS, Wildt et al (1983) found that HR decreased in parallel with pH. This hypothesis could also explain why the lowest HR was found at T3, because this was the time point when metabolic acidosis was more severe. Another possible is that the low HR resulted from increased cytokine levels.

When compared to G2, etomidate-treated animals had lower MAP at T0 (a difference which was statistically significant) and T1. A decrease in blood pressure at T0 was expected based in what was described in other experimental studies.For instances in normal rats, Wilt et al. (1983) showed a decreased MAP following etomidate administration. Similar to our study, in this study etomidate was administered to mechanically ventilated animals which were pre-medicated with an opioid. However, the dose of etomidate was higher (bolus of 5 mg/kg followed by a continuous infusion of 10mg/kg/hr) and the opioid was fentanyl administered by continuous infusion. These differences might explain why Wildt et al. (1983) found a higher and longer decrease in MAP (at the end of the experiment, 4 hours after etomidate administration, MAP decreased from 106 +/-7 mmHg to 55+/- 6 mmHg) (Wildt et al., 1983). The effects of etomidate in MAP in HS models have been more variable. A study in rats submitted to HS also described a decrease in MAP after etomidate (De Paepe et al., 1999). Etomidate administration has also been associated to a decrease in MAP in Humans (Price et al., 1992) and pigs (Prakash et al., 1981). In contrast, this did not occurred in another study in pigs (Johnson et al., 2003), in mice (Paris et al., 2003) and dogs (Pascoe et al., 1992; Wauquier 1983). The different effects of etomidate in blood pressure may be explained by different dose regimes, specie-related differences or the degree of hypotension induced in

the different HS models. Regarding the latter the reasoning is what follows: when the degree of hypotension achieved was severe, a stronger compensatory pressor response would follow. Thus this immediate compensatory response could potientially masked the hypotensive effects of etomidate.

How etomidate decreases MAP is still not completely understood although there are several possibilities. One is that etomidate increases SVR which than interferes with cardiac pump function (Wildt et al., 1983). The increase in SVR seems to be related with direct effects of etomidate in vascular α_{2B}-receptors (Creagh et al., 2010; Paris et al., 2003) and from an etomidate-mediated increase in sympathetic tone (Ebert et al., 1992). Etomidate may also decrease cardiac contractility in a dose-dependent manner (Buljubasic et al., 1996; Gelissen et al., 1996). Although this does not normally occur at clinically used doses, it may become apparent in pathologic conditions associated to increased etomidate´s concentrations such as sepsis (Zausig et al., 2009). In addition it was described that etomidate in high concentrations causes systemic vasodilation (Shirozu et al., 2009). The combination of increased vasodilation and decreased cardiac contractility can lead to a decrease in CI, which further compromises the maintenance of blood pressure. A decrease in CI following etomidate has been found by some authors in anesthetized subjects (Price et al., 1992; Wildt et al., 1983; Prakash et al., 1981) but not in experimental models of HS in swine (Johnson et al., 2003).

In our case, we hypothesize that the initial decrease in MAP at T0 was mainly due to etomidate´s associated increased in SVR and subsequent decreased in cardiac pump function. This lower MAP could in part justify why HS was more easily induced in etomidate-treated animals, as suggested by G3´s higher Δvolume. In contrast, the lower levels of MAP at T1 could represent a true interference of etomidate in the normal compensatory mechanisms to blood loss. In this way, etomidate-treated animals were less effective in restoring blood pressure than animals of G2. Although the way how etomidate promoted this effect was not determined, we hypothesize that this was related to a marked decrease in cardiac contractility and increase in systemic vasodilation. In turn, this could have be related with the effects of HS in etomidate´s pharmacokinetics and pharmacodynamics, increasing its concentration, duration and possibly, potency of action.

The pharmacokinetic profile of plasma etomidate concentration after its administration as a single bolus is best described by a three compartment model. Its decline in plasma is composed by three phases: fast, intermediate, and slow which are thought to correspond, respectively, to distribution into highly perfused tissues (such as the brain), redistribution into peripheral tissues (mostly muscle), and terminal metabolism (Forman, 2011). Etomidate´s terminal metabolism in laboratory animals and Humans rely only in hepatic esterase activity, which hydrolyzes the drug to a carboxylic acid and an ethanol leaving group. Consequently,

etomidate´s metabolism depends from the maintenance of an adequate hepatic blood flow. Several studies have demonstrated that HS induces an increase in etomidate´s concentrations and a decrease in its clearance and distribution volume (Johnson et al., 2003; De Paepe et al., 1999). The decrease in clearance is mainly attributed to decreased liver perfusion whereas the reduction in distribution volume is related with the reduction in circulating blood volume and CO. In addition because the compensatory mechanisms to HS cause a redistribution of blood flow away from less vital organs to the heart and brain, the latter organs become more exposed to etomidate and consequently to its side-effects. Furthermore the redistribution of etomidate´s to peripheral tissues (the intermediate phase of the decline of its concentrations) is delayed, causing a prolongation of its action (De Paepe et al, 1999).

Taking all this together, our hypothesis is that HS potentiated etomidate´s cardiovascular effects to a point that these interfered with the body´s compensatory mechanisms to restore macrocirculation parameters such as MAP. This in turn could have impeded the restoration of tissue perfusion in an adequate manner, aggravating tissue ischemia and subsequent injury. Although plausible, we could not confirm this hypothesis. To confirm it would imply that we measured etomidate´s concentrations and hepatic perfusion (see the section of future perspectives).

Did low CS levels contributed to the macrocirculatory disturbances?

It may be questioned if the “relative deficiency” of CS in etomidate-treated animals played a role in the lower MAP of G3 from T0 to T1. This question is based in several assumptions. First, glucocorticoids are known to have a physiological role in maintaining cardiovascular homeostasis (Sapolsky, Romero & Munck, 2000), especially by regulating adrenergic receptor numbers and responses (Annane et al., 1998). Second, there is abundant evidence in the literature that demonstrates the contribution of glucocorticoids to the compensatory cardiovascular response to HS. This evidence comes both from experimental and clinical studies.

In the experimental setting most studies about this subject were performed in adrenalectomised animals. Darlington et al. (1990) showed that hemorrhage in conscious adrenalectomised rats led to cardiovascular collapse and death, and that this could be partially prevented by infusions of CS to mimic the normal increase of CS during HS. Importantly, the same study also showed that cardiovascular collapse and death were completely prevented if rats had chronic CS treatment before the induction of HS (Darlington et al., 1990). Thus, this study provided evidence that adequate levels of CS must be present before HS in order that its beneficial effects are maximized. The results of this study support

or hypothesis that the animals of G3, by having lower CS levels before HS, would be at higher risk of developing cardiovascular collapse than G2.

Other studies in adrenalectomised animals provided other clues about the cardiovascular beneficial effects of glucocorticoids in HS. Adrenalectomised rats have decreased vascular reactivity which is corrected by the supplementation with glucocorticoids to achieve levels similar to CS´s physiological levels c (Darlington et al., 1989). There is also evidence from adrenalectomised animals that glucocorticoids are important for the restoration of blood volume following HS (Darlington et al., 1997; Gann et al., 1983; Ware et al., 1982). These effects are the result of an increased plasma osmolality promoted by glucocorticoids. The increase in plasma osmolality then increases the movement of fluid from the interstitium and intracellular compartments to the intra-vascular space, restoring blood volume (Gann et al., 1983; Ware et al., 1982; Pirkle & Gann, 1976). The rise in plasma osmolality results from a glucocorticoid-dependent passage of extracellular solutes, such as glucose and Na^+, from the intracellular and interstitium compartments to the vascular space. Thesewill drag fluid with them (Darlington et al., 1997). Besides glucocorticoids are also known to interact with catecholamines in the compensatory response HS (Keel & Trentz, 2005).

Clinical studies also provided evidence for the beneficial role of glucocorticosteroids in HS. A study with Human patients with HS showed that the development of early adrenal dysfunction was associated with increased need of vasopressor therapy (Hoen et al., 2002). Furthermore, hydrocortisone administration increased the sensitivity to α_1-adrenoceptor stimulation in fully resuscitated severe trauma patients following HS (Hoen et al., 2005). If etomidate-associated CIRCI is associated to significant changes in blood pressure is still unresolved. The use of etomidate was associated to increased episodes of hypotension in the first 24 hours after its administration as a single bolus (Banh et al., 2012). However, its administration was not associated to increased need of vasopressor therapy, a surrogate of hemodynamic instability (Basciani et al., 2016; Alday et al., 2014; Elliot et al., 2012).

Against the hypothesis that the relative deficiency of CS played a significant role in our results, there are the results of Peterson and Hoffman (1985). The study did not found differences between the compensatory responses to hypovolemia between etomidate-treated and control animals. However in this study, rats had etomidate administered as a single bolus, at the dosage of 3 mg/kg IV 30 minutes before the induction of HS. Based in what is known about etomidate´s pharmacokinetics, the rats which have received etomidate would no longer be under its hypnotic and cardiovascular effects at the time HS was induced (Freeman, 2011). However, they would still be with adrenal suppression (Freeman, 2011). In other words, the pure adrenal suppressive effects of etomidate did not seem to play a role in the compensatory response to HS. This assumption cannot be confirmed however, because in the study of Peterson and Hoffman (1985), etomidate´s and CS concentrations were not

assessed. Moreover, there are significant differences that limit the comparison between the Peterson and Hoffman (1985) study with our own. In the latter, the animals were not mechanically ventilated and the timing and dosage of etomidate were significantly different.

All this considered, and returning to the original question of this section, the answer is that the relative deficiency of CS might have contributed to a compensatory response to HS which was less effective. In other words if existent, it was mainly manifest in the period from T0 to T1. In the period from T2 to T3 it certainly did not contributed to any decrease in MAP. In fact from T2 to T3, when CS levels were at their lowest level, the MAP was actually higher in G3 than in G2.

The reason why this occured remains unclear. One possibility is that resuscitation increased significantly the circulating blood volume. However if the increase in MAP was only due to volume resuscitation, this would also be apparent in G2. Another possibility is that resuscitation reversed the changes in etomidate´s pharmacokinetics induced by HS. Although this has not been reported for etomidate, it has been described for propofol (Johnson et al., 2004). In this hypothesis, resuscitation increased hepatic perfusion and the distribution volume of etomidate. This would lead to a decrease in its concentrations and consequently to a reversal of cardiac depression and vasodilation. Alternatively, the decreased CS levels led to a compensatory increase in vasoactive substances such as AVP, norepinephrine and renin which increased SVR (Darlington et al., 1990). Finally, these changes could have resulted from the higher levels of ACTH in G3, at T1 and T2. In fact, it has been shown that ACTH administration to rats submitted to HS reverses the shock condition by decreasing NO levels (Guarini et al., 1997) and prevents HS-induced vascular dysfunction (Squadrito et al., 1999).

Interference of etomidate in the microcirculation

Etomidate may have also interfered with tissue DO_2 by affecting the microcirculation. To our knowledge a direct and deleterious effect of etomidate in the microcirculation has never been demonstrated. In normal patients undergoing anesthesia, etomidate did not affect tissue oxygenation (Criado et al., 1983). In a canine study etomidate increased critical DO_2 in a dose–dependent manner, an effect which was mainly related with a decrease in tissue oxygen extraction capabilities (Van der Linden, Schmartz, Gilbart, Engelman & Vincent, 2000). It may be hypothesized that this decrease in tissue oxygen extraction may have contributed to tissue hypoxia if microcirculation dysfunction was already present. This might have been the case because HS is itself associated to microcirculatory disturbances (Keel & Trentz, 2005). Besides the combination of etomidate´s associated macrocirculatory disturbances its pro-oxidant effects and etomidate-associated increase in systemic and

possibly local cytokine levels could have aggravated microcirculatory disturbances. In this way, etomidate might have acted at several levels to induce a vicious and progressive cycle of hypoperfusion, tissue oxygen debt, tissue damage and increased inflammation.

5.3.4. Molecular biology variables

In this study, when comparing both HS groups, etomidate-treated animals had higher plasma levels of *TNF-α, IL-6* and *IL-10* mRNA than G2 animals at all time points, except at T0, when G2 had higher *IL-10*.

Cytokine mRNA quantification by qPCR is widely used to investigate cytokine profiles, particularly in small-sized samples. It is currently considered the most reliable method to quantify low-level transcripts such as cytokine and cytokine receptor mRNAs, to investigate cytokine networks and to analyze cytokine production in biological tissues (Peinnequin et al., 2004). qPCR is considered more sensitive to evaluate the fine-tuning of the immune response than the measurement of serum cytokine levels itself (Peinnequin et al., 2004) because the latter only represents a small part of the total amount of cytokines produced (Cavaillon 1993). mRNA quantification by RT-PCR can also be used to provide information of *in vivo* immune response mechanisms and immune monitoring (Stordeur et al., 2003).

From all types of methodologies based in qPCR, “real-time” PCR (RT-PCR) was the method chosen for this study. RT-PCR is considered the most accurate to investigate cytokine activity. It also provides the results in a much faster rate than other methods of mRNA quantification by PCR, because it does not imply post-PCR manipulations (Giulietti et al., 2000). It has also been demonstrated that mRNA obtained through RT-PCR correlates well with cytokine serum and plasma levels (Alaaeddine, De Montigny & Sadouk, 2011; Hein, Schellenberg, Bein & Hackstein 2001; Blaschke, Reich, Blaschke, Zipprich & Neumann, 2000).

The tissue expression of cytokine mRNA after HS has been assessed in several studies (Liu et al., 2007; Rahat et al., 2001). However to our knowledge, the evaluation of cytokine mRNA in whole blood as been reported less frequently and mostly in other species than the rat (Mao et al., 2009; Biberthaler et al., 2004).

Our findings of increased expression of cytokine mRNA in the blood of animals submitted to HS was expected considering the pathophysiologic mechanisms of this condition. HS is associated to an increased immune response with increased cytokine levels, which involves not only the secretion of preformed cytokines but also its synthesis *de novo*. Because the latter involves an increase in cytokine´s genetic expression, an increase in the levels of their correspondent mRNA is an expected finding. Plasma nucleic acid circulation has also been

hypothesized to be a method of cell communication and to be actively secreted by cells (van der Vaart & Pretorius, 2008). Accordingly, it is possible that our findings may also result from increased secretion by cells as part of the general immune response to HS. The major cell types which could have been involved in the higher mRNA cytokine levels are bloodleukocytes, especially monocytes (Mao et al., 2009; Biberthaler et al., 2004). It may be asked why etomidate-treated animals had higher levels of cytokine mRNA when compared with G2.There are several possible explanations including:

- *Decreased CS levels in etomidate-treated animals*: We believe that this was the main reason which justified increased levels of cytokine mRNA in etomidate-treated animals. Glucocorticoids decrease cytokine expression through transcriptional and posttranscriptional mechanisms (Smoak & Cidlowski, 2006). Once ligated to glucocorticoid receptors, glucocorticoids repress inflammatory gene transcription through a direct protein synthesis-independent process (transrepression), or by activating transcription (transactivation) of multiple anti-inflammatory /repressive factors (King et al., 2013). Examples of cytokines whose expression is decreased by glucocorticoids include TNF-α (King, Holden, Gong, Rider & Newton 2009), and IL6 (Wang et al., 2012).

- *Increased cytokine levels*: The increased levels of some cytokines could have increased the genetic expression of others. For instances increased levels of TNFα stimulate the genetic expression of IL6 and IL10. In our study we found a positive correlation between the genetic expressions of *TNFα*, *IL6* and *IL10*, which corroborates this hypothesis. In addition, some cytokines increase their own expression as well. This has been shown in particular for TNFα (Ikejima et al., 1990; Ulich, Guo, Irwin, Remick & Davatelis, 1990).

- *Direct effects of etomidate in cytokine expression*: Etomidate has been shown to interfere with intra-signaling pathways associated to cytokine expression and translation, such as NF-κB (Zhang et al., 2015). As discussed previously, the effects of etomidate in cytokine production and secretion are complex and vary with the type of experimental model and dosage of etomidate. Although this has not been studied, the same probably occurs with etomidate effects in cytokine genetic expression. Nevertheless, based in our findings, a direct effect of etomidate cannot be completely ruled.

The levels of cytokines and their correspondent mRNA were not correlated in this study, in accordance to what has been published by others (Ulich et al., 1990). This may be explained by several reasons. One possible reason is that at at the time the cytokine was secreted, its correspondent mRNA was no longer present due to degradation. Furthermore cytokine production and secretion is post-transcriptionally regulated (Ulich et al., 1990). This means that even if the adequate post-translational regulation is not met, even if mRNA is produced

in adequate quantities, cytokine production and secretion does not occur (Ulich et al., 1990; Remick et al., 1989).

Both G0 and G1 had evidence of increased cytokine expression. In some cases this was even higher than in HS groups, although statistically significant differences were not found. Nevertheless, increased cytokine genetic expression in G0 and G1 was not unexpected for two reasons. The first is that surgical placement of intravascular catheters is associated to some degree of tissue injury and consequently of inflammation with secondary immune cell activation . The second is that isoflurane anesthesia with mechanical ventilation in rats has been associated to increased gene expression of pro-inflammatory cytokines (Kotani et al., 1999). Curiously, the pattern of cytokine expression differed between G0 and G1, which suggests that buprenorphine could have modulated cytokine expression. Opioid drugs are known to modulate cytokine genetic expression through still incompletely understood mechanisms. Studies have shown that opioid modulation of cytokine expression is influenced by factors such as specie, cell type, nature of the immune stimulus, type of opioid receptor involved and other experimental conditions (Bonnet, Beloeil, Benhamou, mazoit & Asehoune, 2008; Sacerdote, Limiroli & Gaspani 2003). It is known that once the opioid ligates to its receptor(s), a change in the activity of several intra-cellular signaling pathways and transcription factors occurs, leading to changes in gene expression (Sharp, 2006; Mccarthy, Wetzel, Sliker, Eisenstein & Rogers, 2001). Transcription factors which are known to be influenced by opioids include NF-κB and AP-1 (Martin-Kleiner, Balog & Gabrilovac, 2006). To our knowledge few studies have described the influence of buprenorphine in cytokine genetic expression. Recently, it was described that buprenorphine led to different genetic expression in the brain when compared with other μ-receptor agonists (morphine and methadone) (Belkaï et al., 2013). Buprenorphine and morphine were also able to activate different intra-cellular signaling pathways and to influence genetic expression in a distinct way, by different regulation of the G-protein μ receptor coupling (Saidak et al., 2006).

The analysis of molecular biology data was limited by the fact that we could only obtain an accurate quantification of mRNA in a limited amount of samples. Several explanations can be advanced to explain this finding, including both biological and methodological reasons. We will begin with biological explanations first. It is known that in HS, tissue cytokine gene expression is time-dependent (Liu et al*.,* 2007). If the same occurs with blood mRNA, it is possible that if its measurement was performed at other time-pointsthe results would be different. In addition its is possible that their modest levels were a reflection of the mild severity of the model. In addition we cannot rule out that the choice of using Wistar rats might have influenced the results as well, because different strains have different responses to inflammation (Yao, Yang & Hou, 2009; Kjellén, Issazadeh, Olsson & Holmdahl, 1998).

Regarding methodological reasons which could have influenced our results these include factors such as general anesthesia, type of experimental model and the methodology used for obtain mRNA. An important factor is the limited amount of blood volume used to extract mRNA, which might have contributed to the low amount of mRNA available for analysis. wh, Another aspect which deserves discussion was the choice of *beta-actin* as reference gene. To our knowledge, no reference gene has been suggested as the ideal housekeeping gene to quantify whole-blood mRNA in HS. The choice of *beta-actin* was based in several assumptions. *Beta-actin* is a commonly used as the housekeeping gene of control in RT-PCR (Suzuki, Higgins & Crawford, 2000) and it has been used with success for this purpose in studies of HS where tissue cytokine mRNA was quantified by RT-PCR (Yang et al., 2007). Besides in preliminary experiments which we performed in few animals submitted to HS, it was possible to obtain mRNA of *beta-actin* in sufficient quantity that permitted its quantification. There are however, two main pitfalls associated with the use of *beta-actin* as housekeeping gene in this experiment. The first is that its genetic expression varies with experimental conditions (Schmittgen & Zakrajsek, 2000). Besides, it is still unknown how *beta-actin* expression is influenced by the development ofHS. Consequently we cannot rule out our results of mRNA could have been influenced by by the way *beta-actin* was expressed.

In resume our findings regarding molecular biology data should be considered just preliminary. Important methodological issues, such as which is (are) the best reference gene(s) to compare the cytokine mRNA and other technical issues remain to be defined. Nevertheless the study demonstrated the applicability of this method to evaluate the inflammatory response in HS and demonstrated for the first time that blood cytokine mRNA can be modulated by buprenorphine and by the administration of etomidate, when this is administered in the context of HS.

5.3.5. Histological variables-apoptosis and necrosis

Apoptosis

In this study it was found that etomidate-treated animals had a lower apoptotic index than G2 in both the cortex and medulla. In addition, the apoptotic rate did not differed significantly from the apoptotic index of G1, and in the medulla, from G0 as well. This was an unexpected finding. In fact, etomidate-treated animals had conditions which would put them at higher risk of developing cell apoptosis (and also necrosis) including higher levels of cytokines (especially TNF-α), hypoxemia, and increased tissue oxygen debt (discussed previously). It seems that something in etomidate-treated animals had a protective effect against apoptosis. Actually, it must be considered that these protective effects of etomidate in apoptosis might

have been the manifestation of a local and specific phenomenon related with the adrenal gland. In the following sections a discussion about what could have prevented apoptosis in etomidate-treated animals will be performed. The hypothesis that this was a local phenomenon will be discussed later.

Did etomidate have a direct anti-apoptotic activity?

Several anesthetics have been reported to possess anti-apoptotic activity, including thiopental (Roesslein et al., 2008) and sevoflurane (Osman et al., 2012). So, the existence of anti-apoptotic effects by etomidate would be plausible as well. However the available literature about this topic is conflicting.

Most studies suggest that etomidate has mainly pro-apoptotic and not anti-apoptotic effects. Etomidate has been associated to increased apoptosis in murine leukemia cells, by enhancing the levels of cytochrome c, AIF, endonuclease G, caspase-9, caspase-3 and Bax proteins, and by inhibiting the expression of Bcl-xl (Wu et al., 2014). Etomidate was also associated to increased apoptosis of myocardial cells (Xu et al., 2014). Curiously, in this study etomidate-associated apoptosis was more pronounced than I/R injury associated apoptosis. It also promoted endothelial cell´s apoptosis by interacting with advanced glycation end (AGE) products (Wang et al., 2014). Etomidate and AGE products upregulated cytochrome c release, activated caspase 3, and promoted PARP lysis. They acted synergically to promote oxidative stress, decrease mitochondrial respiratory chain complex IV and mitochondrial membrane potential, and to upregulate ROS formation and mitochondrial superoxide. Zhang, Xiong, Jiao, Wang & Zuo, (2010), also demonstrated that etomidate increased NO production and mPTP opening in HL-60 cells (Zhang et al., 2010). The only study which did not showed a pro-apoptotic effect of etomidate was when this was compared with other anesthetics regarding its influence in the apoptosis of human T lymphocytes (Roesslein et al., 2008).

More recently, three studies in rats have provided evidence that etomidate is clearly associated to adrenal gland apoptosis (Liu et al., 2016; Liu et al., 2015; Zhang et al., 2015). Liu et al. (2015) in a study with normal rats demonstrated that etomidate promoted adrenal gland cell´s apoptosis and that this was related with increased oxidative stress and NO production. The authors also demonstrated that the anesthetic was able to modulate several intra-signaling pathways and intra-cellular proteins associated to apoptosis. Interestingly, the effects on apoptosis seemed to be dose-dependent. When etomidate was administered by infusion at 1.2 mg/kg/hr for 6 hours increased the production of NO and in adrenal gland cells decreased the activity of SOD, catalase (CAT) and glutathione-peroxidase (GPx). It also increased the levels of proteins involved in the apoptotic pathway including cleaved caspase

3, cleaved PARP, Bax, iNOS, AKT and CREB and several members of the MAPK family, including JNK, ERK1/2 and m38MAPK. However, when 30 minutes before the infusion of etomidate, the authors gave the anesthetic as a bolus (0.6 mg/kg), the results were the opposite. Etomidate improved oxidative stress by enhancing SOD, CAT and GPx activity and ERK1/2, CREB and Bcl-2 activation. It also reduced NO production and suppressed JNK, iNOS, cleaved-caspase3, PARP, Bax, and AKT activation (Liu et al. 2015). Zhang et al. (2015) described the effects of etomidate at bolus (0.6 mg/kg) or through infusion (2mg/kg/hr for 2 hours) in rats submitted to CLP and evaluated the rate of adrenal gland apoptosis (Zhang et al., 2015). They found that depending from the dosage and time of administration, etomidate could prevent or increase adrenal apoptosis. In addition they found that at low doses (bolus of 0.6 mg/kg), it was able to induce a preconditioning effect.When administered at this dosage and immediately after CLP,it did not increased significantly adrenal apoptosis and was actually associated to decreased TNF-α and IL6 levels, TNF-α mRNA intra-adrenal expression and NF-kB activity. Finally, Liu et al. (2016) also in a CLP model, demonstrated that a bolus of etomidate (2mg/Kg) at 6 hours post-CLP exacerbated sepsis-induced apoptosis in adrenal gland cells and lymphocytes (Liu et al., 2016). In contrast the administration of the same dosage to control animals did not increased significantly apoptosis in lymphocytes or adrenal gland cells. The authors suggested that the pro-apoptotic effect of etomidate, which was than only manifest in septic animals, was due to direct immunotoxicity, ability to increase oxidative stress and enhancing of systemic inflammation, in particularly, of proinflammatory cytokines such as TNF-α.

In summary, these studies suggest that etomidate is able to modulate the development of adrenal gland apoptosis, both directly, by affecting several intra-signaling pathways and intracellular proteins associated to the apoptotic process, and indirectly, by modulating oxidative and nitrosative stress and systemic inflammation. The effects of etomidate in apoptosis seem to depend significantly from the dosage and timing of administration in relation to the development of the pathological condition. For example at high doses, etomidate increases adrenal apoptosis and potentiates CLP-induced apoptosis.

However, when used preemptively, at low dosages, etomidate can induce a preconditioning and beneficial effect.

As stated before, increased ROS and I/R injury are associated to apoptosis in critical illnesses. If we take this in consideration, because etomidate has a pro-oxidant activity (Wang et al., 2014; Yagmurdur et al., 2004) and can increase neutrophil oxidative burst activity (Szekely et al., 2000), this would argue against a protective effect of etomidate against apoptosis. On the other hand, most studies so far have shown that etomidate modulates favorably I/R injury (Ergün et al., 2010; Yu et al., 2010), limiting I/R injury-related apoptosis.

In resume, we hypothesize that in our study etomidate was able to directly modulate adrenal gland cells apoptosis. In addition, we believe that the effects of etomidate were most likely protective, possibly through a preconditioning effect, based in our results and in what has been described in septic models. The preconditioning effect would result from the fact that we used a low dosage of etomidate and immediately before the development of HS. Nevertheless, because HS represents a different pathological condition from sepsis, and the dosage of 1mg/kg is slightly higher than the dose associated to preconditioning in the septic studies (0.6mg/kg) our hypothesis can only be confirmed in future studies.

Etomidate´s indirect effects in adrenal apoptosis

Etomidate could have also decreased apoptosis indirectly through several possibilities, including by:

- Decreasing CS levels;
- Increasing ACTH;
- Increasing IL6 and IL10.

Did decreased CS levels could have contributed to decreased adrenal apoptosis?

This hypothesis is supported by several lines of evidence. Glucocorticoids have pro-apoptotic activity in several types of cells, including thymocytes (Takai et al., 2000), neurons (Crochemore et al*.,* 2005), lymphocytes (Tuckerman et al., 2005), lung epithelial cells (Kamiyama et al., 2008) and pancreatic acinar cells (Zhang et al., 2007). Studies have also associated increased glucocorticoid levels to parenchymal cell apoptosis in burn patients (Fukuzuka et al., 1999) and in experimental models of sepsis (Kamiyama et al., 2008). Furthermore, in rats the administration of dexamethasone increases apoptosis in adrenocortical cells, which is especially marked in *zona reticularis* and the inner half of *zona fasciculata* (Almeida et al., 2006). Although the mechanisms on how glucocorticoids increase adrenal gland apoptosis are still incompletely understood, they seem to involve increased caspase-3 activity (Almeida et al., 2007).
Based on this evidence we hypothesize that another contribution to the decreased apoptotic activity in etomidate-treated rats was the decreased levels of CS. Unfortunately we were not able to identify a significant correlation between apoptotic index and the levels of CS, which would further provide support to our hypothesis.

Did an increase in ACTH levels contributed to decreased adrenal apoptosis?

We believe that the higher levels of ACTH in etomidate-treated rats could have helped to prevent adrenal gland apoptosis, based in several assumptions. ACTH has known anti-apoptotic properties in several cell types, including adrenal gland cells (Keramidas, Feige & Thomas, 2004; Carsia et al., 1996). Besides, ACTH administration completely blocks the development of apoptosis in *zona reticularis* and the inner half of *zona fasciculata*, which occurs following hypophysectomy and dexamethasone administration (Almeida et al., 2007; Almeida et al., 2006; Carsia et al., 1996).

The mechanisms underlying ACTH anti-apoptotic activity are still poorly understood, but they include both direct and indirect mechanisms. ACTH has direct anti-apoptotic properties by its ligation to melanocortin receptors, which subsequently activate the JAK/ERK/STAT pathway (Ottani et al., 2013; Si et al.; 2013). The role of this pathway is particularly important in ACTH prevention of TNF-α-induced apoptosis (Si et al., 2013).

ACTH has also indirect anti-apoptotic effects by decreasing and/or antagonizing pro-apoptotic factors such as hypoxia/ischemia, oxidative stress, I/R injury and increased inflammation. ACTH administration has been shown to decrease ROS production (Guarini et al., 1996), leukocyte adhesion and infiltration into tissues (Bertuglia & Giusti, 2004; Squadrito et al., 1999; Guarini et al., 1996), TNF-α levels (Guarini et al., 2004; Altavilla et al., 1998), iNOS activity (Bazzani, Bertolini & Guarini, 1997) and vascular dysfunction (Bertuglia & Giusti, 2004; Squadrito et al., 1999). Parts of these effects of ACTH seem to be related with the activation of the vagus nerve-mediated brain cholinergic anti-inflammatory pathway. ACTH achieves this through ligation to the MC4 receptor. This leads to suppression of NF-kB and inflammatory cascade activation pathways and reversal of the shock condition (Guarini et al., 2004; Squadrito et al., 1999).

Did increased cytokines levels have any role in apoptosis development in etomidate-treated animals?

As previously discussed, increased systemic inflammation has been associated to development of adrenal apoptosis in several experimental models (Liu et al., 2016; Yu et al., 2016; Kanczkowski et al., 2013c; Yu et al., 2012). Because G3 animals had increased cytokine levels, the most likely answer to the question is that increased inflammation was *pro-apoptotic*. Importantly, etomidate-treated animals had higher levels of TNF-α and this cytokine is pro-apoptotic in many cell types, including adrenocortical cells (Tkachenko et al., 2011; Mikhaylova et al., 2007).

However, the influence of cytokines in apoptosis depends from several factors, including the type of cytokine and its target cell. Cytokines such as IL1, IL6, and granulocyte colony-stimulating factor often inhibit apoptosis (Oberholzer, Oberholzer, Clare-Salzler & Moldawer, 2001). To our knowledge no studies have reported the effects of IL6 or IL10 in adrenal gland apoptosis. Nevertheless, because many studies have attributed anti-apoptotic effects to these cytokines and etomidate-treated animals had increased levels of IL6 and IL10, we hypothesize that their increased levels contributed to our findings. In addition we believe that they had a synergistic effect with etomidate´s and ACTH´s anti-apoptotic effects.

Several studies have demonstrated that IL6 has anti-apoptotic effects in several organs including the liver, intestine, lung and heart (Thacker et al., 2013; Moran et al., 2009; Moran et al., 2008; Rollwagen et al., 1998). The mechanisms behind IL6 anti-apoptotic effects are still incompletely understood but include both direct and indirect effects. IL6 has direct anti-apoptotic effects by activation of STAT3 through the IL-6/gp130/JAK/STAT intracellular-pathway (Moran et al., 2009; Hirano et al., 2000). STAT3 is the critical regulator of IL6-dependent cell growth, differentiation, and survival signals. It promotes the transcription of several pro-survival regulatory genes including c-myc, Bcl-XL, and myeloid cell leukemia 1 (Mcl-1) and inhibits p53 function (Guo et al**.,** 2012). It also upregulates other pro-survival genes such as *Bcl-2*, *Hsp 70* and *Hsp 40* (Thacker et al., 2013; Moran et al., 2009; Rollwagen et al., 1998) and down regulates the pro-apoptotic genes *Bad*, *Bnip3* and *Casp3* in a tissue specific manner (Thacker et al., 2013; Taub, 2003; Levy & Lee 2002; Bromberg et al., 1999). The changes in gene expression are translated by increases in the levels of anti-apoptotic proteins such as Bcl-xL and Bcl-2 (Kovalovich et al., 2001). IL6 also promotes DNA repair by activating DNA repair pathways (Centurione & Aiello, 2016). Examples of IL6´s direct antiapoptotic activity have been reported in several studies. In intestinal cells, IL6 was found to be protective against apoptosis caused by hypoxia and I/R injury (Rollwagen et al., 2006; Rollwagen et al., 1998). This cytokine also reversed stausporine-induced apoptosis in esophageal carcinoma cells (Leu et al., 2003). Furthermore, mice KO for IL6 do not express Bcl-2 and Bcl-xL in the liver. This makes them more susceptible to ethanol- and TNF-α induced apoptotic killing, a susceptibility which is completely reversed by IL6 administration (Hong et al., 2002).

IL6 also counteracts apoptosis by indirect effects, including by decreasing oxidative stress, suppressing cytosolic Ca^{2+} increase, and prevention of mitochondrial dysfunction (Smart et al., 2006; El-Assal et al., 2004).

Most of available literature attributes to IL10 an anti-apoptotic role (Behrendt et al., 2016; Thompson et al., 2013; Dhingra et al., 2011), although there is also evidence for the contrary (Bailey et al., 2006). IL10 has direct anti-apoptotic effects by activating STAT3 (Moore et al., 2001). Through this, IL10 initiates an anti-apoptotic cascade by blocking caspase-3 and

cytochrome c activity (Boyd et al., 2003; Bachis et al., 2001). It is known that STAT3´s activation is fundamental for IL10 prevention of TNF-α-induced apoptosis (Dhingra et al., 2011). IL10´s anti-apoptotic effects are also indirect. One example is through its ability to down regulate TNF-α, ILβ1 and IFNγ (Balasingam et al., 1996; Fuchs et al., 1996; Fiorentino et al., 1991). Another is IL10´s ability to decrease glucocorticoid production (Koldzic-Zivanovic et al, 2006) thus preventing glucocorticoid pro-apoptotic effects. IL10 can also decrease ROS formation (Thompson et al., 2013).

In conclusion, we believe that a combination of protective factors against apoptosis, which included etomidate´s direct anti-apoptotic effects, decreased CS levels, higher levels of ACTH, IL6 and IL10 were able to effectively counteract the deleterious effects of increased pro-apoptotic activity of other inflammatory mediators in particular, of TNF-α.

Did hemodynamic and metabolic factors have any role in apoptosis development in etomidate-treated animals?

Theoretically the results of metabolic and hemodynamic parameters of etomidate-treated animals would put them at higher risk of developing apoptosis than G2. Our main argument to justify why this did not happen is the same which was suggested to explain why increased levels of TNF-α were not associated to increased apoptosis. We believe that the combination of protective factors cited before were also able to counteract the pro-apoptotic effects of hypoperfusion, hypoxemia, ischemia and I/R injury.

An alternative hypothesis, which can also be applied in the previous section, is that the protective effects against apoptosis identified in etomidate-treated animals were could be a manifestation of a local phenomenon at the adrenal gland. In other words, it was not a widespread phenomenon. If this hypothesis is true, the decreased adrenal apoptosis in G3 can be more easily conciliated with our results of increased hypoperfusion and systemic inflammation and its known pro-apoptotic effects.

What could then explain why the anti-apoptotic effects predominated at the adrenal? One reason is that different organs may possess different susceptibility to apoptosis promoted by a particular stimulus In fact the induction of apoptosis is dependent upon many factors, including genotypic and phenotypic characteristics of individual cells, and also from the specific environmental milieu that surrounds those cells (Grinnell et al., 2012). For example it has been demonstrated that the susceptibility of cardiac and lung endothelial cells to oxidative stress-induced apoptosis is different (Grinnell et al., 2012). Furthermore, in a CLP model, it was shown that the effects of NF-kB activation in apoptosis are highly tissue/cell-specific (Joshi et al., 2002). Another example comes from the activity of glucocorticoids in apoptosis. In fact these can be pro-apoptotic or anti-apoptotic, depending from where they

act (Almeida et al., 2007; Motoyama et al., 2003). Studies have also shown that this different organ susceptibility to apoptosis also occurs in HS. Organs which are known to be severely affected by necrosis and apoptosis following HS include liver, GI tract, kidney, heart, lung and spleen (Deng et al., 2016; Moreira et al., 2016; Zhou et al., 2015). Interestingly, some of these organs are also those which display high levels of local *TNFα* mRNA production following HS (Liu et al., 2007; Rahat et al., 2001).

To confirm this hypothesis histopathological analysis should had also been performed in other organs. This will be discussed with more detail in the section of future perspectives.

Did adrenal gland apoptosis contributed to CIRCI´s in etomidate-treated animals

As discussed previously we believe that the major contributor for CIRCI in G3 animals were etomidate´s adrenal suppressive effects. However because adrenal apoptosis has been directly associated to CIRCI, inclusively in rats treated with etomidate (Liu et al.¸ 2016; Liu et al.¸ 2015), we believe that apoptosis also contributed to apoptosis in this experiment.

Etomidate effects in adrenal gland necrosis

Etomidate-treated animals had also a lower necrosis score than G2. We are unaware of any study that has reported a protective effect of etomidate in adrenal cell necrosis or in any other cell type. A possible hypothesis to explain these findings is that the same factors that prevented apoptosis also prevented necrosis.

This hypothesis is plausible because apoptosis and necrosis share many pathophysiological mechanisms. Both can be triggered by oxidative and nitrosative stress, increased cytokine levels, DAMPs, I/R injury, Ca^{2+} overload, hypoxia and DNA damage (Vanlangenakker et al., 2008). In addition, our results support the hypothesis that a common mechanism induced necrosis and apoptosis. First, both processes coexisted in the adrenals of the animals submitted to HS. Second the cortical apoptotic rate was positively correlated with the rate of necrosis.

Therefore we hypothesize that the increased levels of ACTH, IL6 and IL10 might have attenuated necrosis by the same combination of mechanisms discussed in the previous section. Eventually it is also possible that etomidate had a protective role, although the mechanisms for this remain speculative.

6. Limitations of the study

This study had several limitations which will be briefly described. One limitation is that the model we chose had several characteristics that make its applicability for the clinical setting limited. The first of these characteristics is that etomidate was administered before HS induction. In the clinical setting, etomidate is normally administered to patients who are in HS or who had this condition before. This means that when etomidate is administrated, the patient is already under the systemic compensatory inflammatory and endocrine responses. In that case the effects of etomidate might be different from the ones we reported here. Moreover, clinical patients rarely present solely with HS. Instead it is quite common that they appear with concomitant traumatic injuries and other comorbidities. These may influence the pathophysiology of HS itself. Although in this experiment, surgical instrumentation is associated to some degree of tissue injury and is actually considered a minor trauma, the extrapolation of these results to traumatic patients with HS is also limited.

Another limitation is that the model we used was of mild severity. Although it is reasonable to assume that in severer cases of HS, the results will be in the same direction but potentiated, this remains unproven.

We also evaluated CIRCI based solely in CS levels. Although this is a simple and accessible method and it advised to diagnosis CIRCI by current guidelines (Marik et al., 2009), it has its limitations and provides limited information regarding the pathophysiology of this condition.

Another limitation is related with the histopathologic variables. In this study apoptosis assessment was made by a combination of TUNEL assay and active caspase-3 immunohistochemistry. Necrosis was solely assessed by histopathological analysis. Although these methods are useful to confirm the presence of necrosis and apoptosis, they provide limited information about its underlying mechanisms and their relation with etomidate, CIRCI´s and adrenal inflammation.

Some of the limitations associated with molecular biology data were already mentioned and discussed in the corresponding section. In this regard, we believe that several refinements should be performed to improve our data collection (see next section).

We also used general anesthesia with isoflurane and animals were pre-medicated with buprenorphine. This means that we cannot completely rule out that our findings were influenced by these drugs. In addition the mechanisms on how buprenorphine exerts its effects were not clarified.

In this model we only used young male rats from the same strain (Wistar). It is known that the response to HS is strongly influenced by the sex, strain and age of animals (Klemcke et al., 2011; Mees et al., 2008). Specie-associated differences can also occur. A recent study demonstrated that clinical sepsis induces inflammation, necrosis and hemorrhage of the

adrenal cortex in human patients, but not in mice submitted to CLP or LPS administration (Polito et al., 2010). Consequently, it cannot be ruled out that our findings are specific to this experimental model.

Finally the number of animals which was used was limited due to ethical reasons. The low number of animals can partially explain the lack of statistical significance in some of the results.

7. Future perspectives

To improve the applicability of this model to the clinical setting we suggest to perform future studies where etomidate is administered during or after HS and eventually associated with a further insult (e.g. head trauma; limb fracture) (Tsukamoto & Pape, 2009). These studies should also include the use of HS with different degrees of severity. Furthermore to clarify the hemodynamic and metabolic effects of etomidate, etomidate should be tested in different doses. The studies should also include the measurement of etomidate´s and hormone concentrations (AVP, angiotensin II, renin, catecholamines, endothelin), assessment of blood flow to specific organs (liver, GI tract, kidney, heart and brain) and eventually evaluation of microcirculation (for example with the measurement of tissue oxygen tension levels). The creation of another group similar to G1 but also with etomidate might also be considered, to further clarify the mechanisms of etomidate in several variables.To better clarify the pathophysiology of CIRCI in HS we believe that further tests should be performed. These may include the measurement of free CS and glucocorticoid receptor concentrations. These tests have shown promise in several studies (Bhatia, Muraskas, Janusek & Mathews, 2014; Moraes, Czepielewski, Friedman & Borba, 2011). In addition, the relationship of HS with CIRCI and adrenal gland pathology should be more thoroughly evaluated, in particular its relation with intra-adrenal inflammation, apoptosis and necroptosis.

In order to evaluate CIRCI relation with adrenal inflammation the measurement of intra-adrenal cytokine concentrations and cytokine mRNA by RT-PCR could be helpful in this regard. The latter can be compared with systemic cytokine and cytokine cmRNA levels to verify if there is any correlation. To further clarify the contribution of specific inflammatory mediators and its mechanisms in CIRCI and adrenal apoptosis development, we suggest to use KO animals for specific mediators (e.g. IL10), the use of small interfering RNA (siRNA) to interfere with specific gene expression and the use of inhibitors of specific intra-signaling pathways (e.g. gp130/JAK/STAT) or intracellular mediators (e.g. STAT3). For example if IL6 and IL10 are prevented of exerting its effects in the adrenal gland, we can assess if they were associated with an anti-apoptotic effect, as proposed.

To provide evidence that apoptosis and necroptosis were associated to CIRCI, we suggest to perform studies which include the evaluation of specific mediators and genes involved in the intra-cellular signaling pathways involved in apoptosis and necroptosis. Examples of these are apoptosis-related genes such as *Bax*, *Bcl2* (and *Bax*/*Bcl2* ratio) *Hsp 70*, *Hsp 40 RIPK1* and *RIPK3*. Intracellular molecules such as RIPK1, RIPK3, Bcl-2, Bcl-xL, AKT, CREB, cytochrome c and others may be considered as well. On a future stage it might be considered the use of drugs that prevent apoptosis and necroptosis, such as cyclosporine, caspase inhibitors and necrostatin.

Regarding etomidate´s role in apoptosis, necroptosis and CIRCI the above techniques can prove to be helpful as well. We suggest using the approaches described by Liu et al. (2015) and Liu et al., (2016) but this time in rats submitted to HS. Particular relevance should be given to the use of different dosages of etomidate and its administration at different time points. This will permit to confirm that etomidate has also a preconditioning effect in HS and that its underlying mechanisms are similar to those which were described in sepsis.

In this study etomidate was also associated to lung injury but this was not confirmed by histopathological analysis. In addition, the underlying mechanisms remain unclear. Thus we suggest that ALI should be confirmed by other diagnostic tests including such as lung histopathological analysis, measurement of bronchoalveolar lavage fluid´s protein and cytokine concentration, and lung neutrophil sequestration and myeloperoxidase activity. To confirm that etomidate was associated to increased organ injury and modulation of necrosis and apoptosis in other organs than the adrenal, we suggest that future studies should evaluate the presence of apoptosis and necrosis (especially necroptosis) in other organs. This may include the lung, heart, intestine, liver, kidney, brain and spleen. Besides, the evaluation of these organs should include assessment of tissue cytokine and cytokine mRNA levels.

Regarding molecular biology data we suggest to use the combination of several reference genes which may or not include *beta-actine*. To investigate the simultaneous expression of several cytokine genes, we suggest using microarray technology due to the amount of information that it gives. In addition we also can evaluate for the presence of cell free mRNA (Etheridge et al., 2013;Ren et al., 2013; Cai et al., 2011; Lam, Rainer, Chan, Joynt & lo, 2003; Zhang et al., 2008; Wong & Lo, 2003; Lo et al., 2000). This will permit a better understanding of the immune response to HS in terms of genetic expression. To verify if etomidate was indeed associated to increased mRNA production, studies which include the exposure of etomidate at different concentrations to activated leukocytes and endothelial cells or whole blood should also be considered.

To rule out the effects of other anesthetic combinations, we suggest to perform studies where etomidate is compared with other anesthetic (e.g. ketamine or propofol) in animals with buprenorphine and isoflurane.

Finally to improve our understanding about the nature of buprenorphine in these setting, we suggest to measure buprenorphine concentrations and to use it in combination with drugs with specific antagonistic activity to different opioid receptors, taking in consideration buprenorphine´s specific pharmacology.

8. Conclusion

In our study we were able to confirm only part of our initial hypotheses. It was demonstrated unequivocally that HS is associated to adrenal gland apoptosis. In addition HS was associated to development of adrenal dysfunction to some degree. Although a clear relation between both events was not found these findings call up for more studies to address this subject. The finding that HS is associated to adrenal apoptosis may have therapeutic implications which range from drugs that can prevent adrenal inflammation and modulate effectively the apoptotic response to early support of adrenal function with glucocorticoids.

Etomidate was associated to development of CIRCI in this model. Thus, we were able to use etomidate to induce a state of "artificial CIRCI". In our opinion, by providing a research model to study CIRCI, the use of etomidate can greatly contribute to advances in our knowledge about this condition.

Additionally it was demonstrated that etomidate-associated CIRCI was associated to development of significant morbidity. The implications of this finding extend beyond the research setting. In fact the use of etomidate is controversial in several settings, namely in sepsis. Our findings suggest that in HS too, the use of etomidate should be used with caution. Future studies should be undertaken to confirm if our findings also occur in critically ill patients and what are their consequences.

Our study also demonstrated two remarkable findings related to etomidate administration. The first is that etomidate was associated to ALI. To our knowledge this makes our study the first experimental evidence of a phenomenon which, until now, has been only reported in clinical patients: the development of lung complications in critically ill patients treated with etomidate. This reinforces the assumption that our research provided a model which can be used in future investigations to study this condition. The second was the finding that etomidate can modulate the occurrence of adrenal apoptosis and necrosis. Although the effects of etomidate in adrenal apoptosis had been previously described in sepsis, to our knowledge this is the first time that they are described in HS. Furthermore, no study had described before that etomidate attenuated the development of adrenal gland necrosis. When future studies clarify the mechanisms on how etomidate exerts these actions, it might be possible to explore etomidate´s modulating effects in apoptosis and necrosis therapeutically.

As a final point, the use of buprenorphine was shown to be associated with significant effects in several variables. This effectively reminds us that all drugs are associated to significant side-effects and that buprenorphine is no exception. Future studies should be undertaken in order to confirm the impact of buprenorphine´s side-effects in HS, when compared with other analgesics.

In conclusion, our study permitted to confirm some of our hypothesis and definitively improved our knowledge regarding HS-associated CIRCI. However most importantly, it opened new lines of investigation with potential significant implications in both the experimental and clinical settings. If adequately explored, we believe that the future studies based on these new lines of investigation have the potential to significantly change the management of patients which are or were in HS and are critically ill.

9. Bibliografia

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10. Addendum

In the following sections the tables mentioned in the results will be displayed.

Table 2: Variation of hormonal variables from T0 to T3 in G0, G1, G2 and G3.

Variables (sample dimension)	***Time***	***G0***	***G1***	***G2***	***G3***
ACTH (pg/ml) (6 < n < 8)	T0	0.00 (0.00-15.20)	18,93 (10.39-33.77)	8,20 (6.69-17.57)	29,70 (18.47-42.87)
	T1	0.00 (0.00-0.00)	9,29 (6.06-18.14)	76,36 (28.28-93.92)	57,01 (39.72-95.75)
	T2	0.00 (0.00-15.20)	9,91 (4.75-21.03)	14,53 (9.90-22.96)	21,87 (11.38-40.68)
	T3	0.00 (0.00-0.00)	22,70 (10.04-34.27)	15,78 (11.80-36.16)	16,96 (10.57-29.87)
Costicosterone (pg/ml) (6 < n < 13)	T0	100755.3 (59803.6-131288.1)	167122,14 (96116,07-224427,81)	126830,91 (87384,09-198382,13)	92250,55 (78542,70-128269,16)
	T1	66012.0 (47666.0-99543.7)	91033,17 (54050,20-172971,10)	262017,35 (205282,33-302860,48)	92503,55 (83669,19-141892,11)
	T2	57865.5 (56836.4-92385.7)	124125,31 (81853,85-201986,60)	144758,30 (98672,32-204686,25)	89045,22 (63792,08-96656,24)
	T3	46314.1 (45042.3-114581.2)	208977,70 (124723,40-271225,61)	115389,10 (96066,48-167478,09)	103557,30 (75296,86-105495,9)

Table 2. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. All data are reported as median and interquartile range (IQR). ACTH: adrenocorticotropic hormone; CS: Corticosterone.

Table 3: Variation of immunological variables from T0 to T3 in G0, G1, G2 and G3.

Variables (sample dimension)	***Time***	***G0***	***G1***	***G2***	***G3***
IL-6 (pg/ml) (4 < n < 14)	T0	458.30 (74.97-800.05)	382.94 (46.75-492.59)	454.04 (354.11-1462.60)	1083.75 (119.88-2890.09)
	T1	374.00 (124.12-909.30)	396.89 (140.02-496.61)	538.84 (374.17-1209.70)	1865.73 (900.79-2884.40)
	T2	372.54 (120.91-959.64)	331.04 (105.43-404.29)	653.26 (345.40-999.26)	2205.24 (1595.83-3397.95)
	T3	381.85 (115.14-824.64)	322.18 (140.01-383.57)	680.84 (632.40-759.17)	3522.47 (2183.75-6905.66)
IL-10 (pg/ml) (5 < n < 14)	T0	0.52 (0.52-0.52)	0.52 (0.52-0.52)	45.03 (0.00-577.04)	540.88 (18.45-784.73)
	T1	0.52 (0.52-0.52)	0.52 (0.52-4.18)	510.89 (73.78-1284.68)	926.74 (51.84-1782.00)
	T2	0.52 (0.52-0.52)	9.20 (0.52-85.75)	1112.55 (559.53-1604.02)	1365.10 (131.55-2450.73)
	T3	0.52 (0.52-0.52)	0.52 (0.52-69.24)	610.39 (0.00-1019.20)	1049.90 (63.49-2887.30)
TNF-α (pg/ml) (5 < n < 14)	T0	0.48 (0.48-0.48)	2.10 (2.10-2.10)	2.74 (2.74-2.74)	11.98 (0.00-106.10)
	T1	0.48 (0.48-0.48)	2.10 (2.10-2.10)	2.74 (2.74-3.01)	119.65 (11.98-151.50)
	T2	0.48 (0.48-0.48)	2.10 (2.10-2.10)	2.74 (2.74-2.85)	79.30 (31.84-106.10)
	T3	0.48 (0.48-1.19)	2.10 (2.10-2.10)	2.74 (2.74-2.74)	95.06 (11.98-196.90)

Table 3. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. All data are reported as median and interquartile range (IQR). TNF-α: Tumor Necrosis Factor-α; IL6: Interleukin 6; IL10: Interleukin 10.

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Table 4: Variation of metabolic variables from T0 to T3 in G0, G1, G2 and G3

Variables (sample dimension)	***Time***	***G0***	***G1***	***G2***	***G3***
pH (12 < n < 16)	T0	7.365 (7.313-7.372)	7.346 (7.262-7.386)	7,270 (7.250-7.339)	7,287 (7.254-7.377)
	T1	7.361 (7.335-7.388)	7.405 (7.313-7.433)	7,291 (7.233-7.346)	7,287 (7.236-7.363)
	T2	7.356 (7.315-7.400)	7.418 (7.346-7.440)	7,295 (7.247-7.316)	7,272 (7.252-7.360)
	T3	7.329 (7.300-7.368)	7.400 (7.365-7.439)	7,309 (7.223-7.339)	7,263 (7.236-7.298)
PO_2 (mmHg) (13 < n < 15)	T0	399 (342-468)	499 (459-537)	510 (393-524)	410 (313-459)
	T1	352 (297-422)	479 (385-518)	410 (361-494)	390 (340-410)
	T2	329 (278-387)	458 (388-513)	451 (328-481)	262 (227-388)
	T3	378 (319-407)	483 (318-520)	447 (216-528)	252 (135-431)
BE (mEq/L) (13 < n < 16)	T0	6 (4-6)	7 (6-9)	5 (5-8)	5 (3-6)
	T1	6 (3-7)	7 (6-8)	1 (-2;4)	-1 (-8;1)
	T2	4 (3-6)	7 (6-8)	2 (1-3)	-1 (-4;0)
	T3	3 (-3;6)	7 (5-8)	2 (-5;3)	-2 (-5;-1)
HCO_3 (mEq/L) (13 < n < 16)	T0	31.6 (29.5-32.3)	32.8 (31.4-35.3)	32,6 (31.8-35.0)	31,2 (30.0-33.0)
	T1	30.9 (28.1-31.6)	32.0 (29.5-32.9)	27,7 (24.9-30.0)	26,2 (18.6-27.5)
	T2	29.1 (27.9-31.0)	32.4 (31.2-33.4)	28,3 (26.3-29.6)	24,3 (23.1-27.2)
	T3	28.5 (23.4-30.0)	31.6 (28.4-32.0)	27,7 (22.6-31.2)	23,9 (21.8-26.0)
Lactate (mmol/L) (8 < n < 13)	T0	1.17 (0.89-1.22)	1.38 (1.04-1.68)	1,06 (0.68-1.20)	1,09 (0.86-1.33)
	T1	1.08 (0.91-1.51)	1.59 (1.46-1.85)	2,37 (1.58-3.40)	2,80 (1.96-5.21)
	T2	1.53 (1.22-1.84)	1.24 (0.88-1.68)	1,48 (1.26-1.62)	1,79 (0.93-2.67)
	T3	1.24 (0.97-1.78)	1.06 (0.83-1.39)	1,03 (0.82-2.38)	1,30 (1.04-3.79)
PCO_2 (mmHg) (13 < n < 16)	T0	55.3 (51.9-58.9)	57.3 (52.8-68.6)	69,8 (60.4-75.3)	65,0 (51.9-74.9)
	T1	53.6 (45.8-56.6)	49.2 (46.1-67.6)	55,3 (48.2-67.5)	53,1 (38.2-62.8)
	T2	51.6 (44.2-59.5)	50.9 (47.4-59.3)	58,3 (49.1-65.1)	53,9 (45.0-56.8)
	T3	49.5 (45.5-58.1)	49.5 (46.4-56.4)	55,1 (45.2-59.8)	50,3 (42.6-60.6)
PO_2 / FiO_2 (13 < n < 15)	T0	399 (342-468)	499 (459-537)	510 (393-524)	410 (313-459)
	T1	352 (297-422)	479 (385-518)	410 (361-494)	390 (340-410)
	T2	329 (278-387)	458 (388-513)	451 (328-481)	262 (227-388)
	T3	378 (319-407)	483 (318-520)	447 (216-528)	252 (135-431)

Table 4. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. All data are reported as median and interquartile range (IQR).

Table 5: Variation of hemodynamic variables from T0 to T3 in G0, G1, G2 and G3

Variables (sample dimension)	Time	G0	G1	G2	G3
Heart Rate (n = 5)	T0	400 (313 -408)	372 (267 - 381)	445 (413 - 455)	372 (205 - 419)
	T1	354 (352 – 368)	389 (361 - 395)	419 (370 - 425)	335 (323 - 363)
	T2	349 (333 – 370)	424 (402 - 432)	404 (373 - 408)	290 (163 - 361)
	T3	360 (298 – 363)	317 (272 - 400)	371 (376 - 383)	233 (154 - 233)
MAP (n = 5)	T0	150 (142 - 153)	152 (140 - 182)	190 (173 - 190)	125 (105 - 125)
	T1	117 (115 – 122)	141 (122 - 144)	135 (129 - 146)	112 (101 - 120)
	T2	93 (87 - 96)	103 (70 - 135)	76 (75 - 94)	103 (101 - 120)
	T3	101 (91 - 117)	79 (75 - 80)	67 (63 - 96)	80 (47 - 105)

Table 5. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. All data are reported as median and interquartile range (IQR).

Table 6: Variation of molecular biology variables from T0 to T3 in G0, G1, G2 and G3.

Variables (sample dimension)	***Time***	***G0***	***G1***	***G2***	***G3***
Log IL-6 (2 < n < 7)	T0	20.00 (16.61-24.57)	7.22 (-4.19;18.64)	5.33 (3.61-19.07)	18.65 (17.47-21.00)
	T1	20.18 (16.71-25.08)	21.07 (16.41-23.63)	19.21 (5.88-22.10)	20.08 (17.81-23.63)
	T2	21.63 (18.82-25.55)	20.07 (19.56-20.58)	18.00 (11.06-20.75)	16.97 (16.39-17.09)
	T3	18.28 (15.36-20.18)	14.39 (11.70-20.84)	19.80 (4.60-21.43)	16.50 (16.47.18.00)
Log IL-10 (2 < n < 7)	T0	13.38 (6.50-19.06)	21.11 (17.60-24.62)	19.21 (19.07-20.84)	18.65 (17.47-21.00)
	T1	20.18 (16.71-25.08)	18.14 (4.87-22.97)	4.88 (-0.94;19.21)	20.08 (17.81-23.63)
	T2	18.82 (12.62-23.99)	18.96 (18.47-19.44)	19.35 (9.55-21.37)	16.97 (16.39-17.09)
	T3	9.56 (-7.00;16.51)	4.07 (2.84-5.30)	1.50 (-1.81;19.80)	16.47 (10.64-18.00)
Log TNF-α (2 < n < 8)	T0	-8.95 (-9.64;47.74)	-2.59 (-6.75;0.70)	0.04 (-7.13; 20.10)	18.83 (10.33-20.00)
	T1	-4.72 (-7.45;-1.46)	-4.65 (-5.28;4.54)	-2.13 (-4.50; 0.93)	20.03 (3.13-23.57)
	T2	-3.62 (-7.29;11.89)	-3.69 (-3.92;-3.24)	-1.93 (-4.09; -0.33)	10.44 (2.93-16.98)
	T3	-4.23 (-7.00;1.17)	-4.11 (-5.61;-3.41)	-1.96 (-4.26; -0.45)	2.73 (1.53-3.57)

Table 6. G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. All data are reported as median and interquartile range (IQR).

Table 7: Variation of apoptotic index in adrenal cortex and medulla and necrosis score in G0, G1, G2 and G3.

Groups	***Cortical*** ***(13≤ n ≤18)***	***Medula*** ***(12≤ n ≤18)***	***Necrose*** ***(9≤ n ≤17)***
G0	2,253 (1.916-4.178)	1,086 (0.439-1.689)	0 (0-0)
G1	4,671 (2.720-5.682)	1,001 (0.570-1.342)	0 (0-0)
G2	7,403 (6.304-8.027)	4,305 (1.426-5.097)	2 (1-2)
G3	5,179 (2.748-8.678)	1,130 (0.503-2.116)	0 (0-2)

Table 7.G0: control animals with general anesthesia and mechanical ventilation only; G1: control animals with general anesthesia, mechanical ventilation and buprenorphine; G2: similar to G1 but also subjected to shock; G3: similar to G2 but also with etomidate administration. All data are reported as median and interquartile range (IQR).

Table 8: Correlations between hormonal, immunological, hemodynamic and molecular biology variables in G1.

Variables	***ACTH (n=29)***		***Corticosterone (n=29)***		***IL6 (n=28)***		***IL10 (n=28)***		***TNF- α (n=28)***		***LogIL10 (n=9)***	
	Spearman r	*p value*	*Spearman r*	*p value*	*Spearman r*	*p value*	*Spearman r*	*p value*	*Spearman r*	*p value*	*Spearman r*	*p value*
Hormonal												
ACTH (n=29)	**-**	**-**	**0.520**	**0.004**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Corticosterone (n=29)	**0.520**	**0.004**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Immunological												
IL6 (n=28)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-0.607**	**0.001**	**-**	**-**
IL10 (n=28)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-0.531**	**0.004**	**-**	**-**
TNF-*α* (n=28)	**-**	**-**	**-**	**-**	**-0.607**	**0.001**	**-0.531**	**0.004**				
Hemodynamic												
HR (n=8)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-0.821**	**0.025**	**-**	**-**
MAP (n=8)	**-0.812**	**0.027**	**-**	**-**	**-**	**-**	**-0.812**	**0.027**	**-**	**-**	**-**	**-**
Molecular Biology												
Log TNF-α (n=9)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**0.714**	**0.043**
LogIL6	**-**	**-**	**-**	**-**	**-0.821**	**-0.821**	**-**	**-**	**-**	**-**	**-**	**-**

Correlation analysis between variables was performed with the Spearman rank correlation, considering the complete set of four time points. Statistical significance was set at a $p < 0.05$.

Table 9: Correlations between metabolic variables with itself and with immunological variables in G1.

Metabolic variables	***HCO_3 (n=60)***		***PCO_2 (n=60)***		***IL6 (n=28)***	
	Spearman r	*p value*	*Spearman r*	*p value*	*Spearman r*	*p value*
pH (n=60)	**-0.418**	**0.001**	**-0.732**	**0.000**		
Lactate (n=32)	**-0.330**	**0.066**	**-**	**-**	**0.515**	**0.005**
BE (n=60)	**0.903**	**0.000**	**0.522**	**0.000**	**-**	**-**
HCO_3 (n=60)	**-**	**-**	**0.749**	**0.000**	**-**	**-**
PO_2 (n=60)	**-**	**-**	**0.319**	**0.013**	**-**	**-**

Correlation analysis between variables was performed with the Spearman rank correlation, considering the complete set of four time points. Statistical significance was set at a $p < 0.05$.

Table 10: Correlations between hormonal and immunological variables in G2 and G3.

		***ACTH* (G2: n= 24; G3: n=52)**		***Corticosterone* (G2: n=24; G3: n=52)**		***TNF-α* (G2: n=24; G3: n=56)**		***IL6* (G2: n=24; G3: n=56)**		***IL10* (G2: n=24; G3: n=56)**	
Group	***Hormonal variables***	**Spearman *r***	***p*-value**	**Spearman *r***	***p*-value**	**Spearman *r***	***p*-value**	**Spearman *r***	***p*-value**	**Spearman *r***	***p*-value**
G2	ACTH (n=24)	**-**	**-**	**0.506**	**0.012**	**0.409**	**0.048**	**-**	**-**	**0.503**	**0.013**
G3	ACTH (n=52)	**-**	**-**	**0.444**	**0.001**	**-**	**-**	**-**	**-**	**-**	**-**
	Immunological variables										
G2	IL10 (n=24)	**0.503**	**0.013**	**-**	**-**	**0.524**	**0.009**	**-**	**-**	**-**	**-**
	TNF-*α* (n=56)	**0.409**	**0.048**	**-**	**-**	**-**	**-**	**-**	**-**	**0.524**	**0.009**
G3	TNF-*α* (n=56)	**-**	**-**	**-**	**-**	**-**	**-**	**0.558**	**0.000**	**0.706**	**0.000**
	IL10 (n=56)	**-**	**-**	**0.298**	**0.032**	**0.706**	**0.000**	**0.575**	**0.000**	**-**	**-**

Correlation analysis between variables was performed with the Spearman rank correlation, considering the complete set of four time points. Statistical significance was set at a $p < 0.05$.

Table 11: Correlations between hormonal and immunological with metabolic and hemodynamic variables in G2 and G3.

		ACTH (G2: n= 24; G3: n=52)		*Corticosterone (G2: n=24; G3: n=52)*		*TNF-α (G2: n=24; G3: n=56)*		*IL-6 (G2: n=24; G3: n=56)*		*IL-10 (G2: n=24; G3: n=56)*	
Group	***Metabolic variables***	***Spearman r***	***p-value***	***Spearman r***	***p-value***	***Spearman r***	***p-value***	***Spearman r***	***p-value***	***Spearman r***	***p-value***
G2	pH (n=52)	**-**	**-**	-	-	**-0.463**	**0.024**	-	-	-	-
	HCO_3 (n=52)	**-0.566**	**0.004**	-	-	-	-	-	-	**-0.418**	**0.043**
	BE (n=52)	**-0.572**	**0.004**	-	-	-	-	-	-	**-0.440**	**0.032**
	Lactate (n=52)	**0.678**	**0.000**	**0.490**	**0.016**	-	-	-	-	**0.422**	**0.041**
	PCO_2 (n=52)	**-**	**-**	-	-	**-0.601**	**0.002**	-	-	-	-
G3	pH (n=52)	**-**	**-**	-	-	**-0.435**	**0.001**	**-0.325**	**0.016**	**-0.500**	**0.000**
	HCO_3 (n=52)	**-**	**-**	-	-	**-0.464**	**0.000**	**-0.466**	**0.000**	**-0.444**	**0.001**
	BE (n=52)	**-**	**-**	-	-	**-0.548**	**0.000**	**-0.502**	**0.000**	**-0.548**	**0.000**
	Lactate (n=52)	**0.300**	**0.031**	-	-	-	-	-	-	-	-
	Hemodynamic variables										
G2	HR (n=8)	-	-	-	-	-	-	-	-	-	-
	MAP (n=8)	-	-	-	-	-	-	-	-	-	-
G3	HR (n=16)	-	-	-	-	-	-	-	-	-	-
	MAP (n=16)	-	-	-	-	-	-	-	-	-	-

Table 12: Correlations between metabolic variables itself and molecular biology variables in G2.

Metabolic variables	***HCO_3 (n=52)***		***Lactate (n=52)***		***pH (n=52)***		***Log IL-6 (n=22)***	
	Spearman *r*	*p* value	Spearman *r*		Spearman *r*	*p* value	Spearman *r*	*p* value
BE (n=52)	**0.886**	**0.000**	**-0.424**	**0.002**	**-**	**-**	**-**	**-**
HCO_3 (n=52)	**-0.556**	**0.000**	**-0.556**	**0.000**	**-**	**-**	**-**	**-**
PCO_2 (n=52)	**-0.556**	**0.000**	**-0.489**	**0.000**	**-0.686**	**0.000**		
PO_2 (n=52)	**-**	**-**	**-**	**-**	**-**	**-**	**-0.439**	**0.043**

Correlation analysis between variables was performed with the Spearman rank correlation, considering the complete set of four time points. Statistical significance was set at a $p < 0.05$.

Table 13: Correlations between metabolic variables itself in G3.

Metabolic variables	***HCO_3 (n=55)***		***Lactate (n=52)***		***PCO_2 (n=56)***	
	Spearman *r*	*p* value	Spearman *r*	*p* value	Spearman *r*	*p* value
pH (n=56)	**-**	-	**-**	-	**-0.634**	0.000
BE (n=55)	**0.957**	0.000	**-0.643**	0.000	**0.578**	0.000
HCO_3 (n=55)	**-**	-	**-0.670**	0.000	-	-
PCO_2 (n=56)	**0.758**	0.000	**-0.594**	0.000	-	-
PO_2 (n=56)	0.044	0.750	**0.321**	0.020	-	-

Correlation analysis between variables was performed with the Spearman rank correlation, considering the complete set of four time points. Statistical significance was set at a $p < 0.05$.

Table 14: Correlations between immunological and molecular biology variables in G3.

	Log TNF-α (n=15)		***Log IL6 (n=12)***		***IL6 (n=56)***	
Molecular Biology variables	*Spearman r*	*p value*	*Spearman r*	*p value*	*Spearman r*	*p value*
Log TNF-α(n=15)	**-**	**-**	**0.874**	**0.000**	**-**	**-**
Log IL6 (n=12)	**0.874**	**0.000**	**-**	**-**	**-0.762**	**0.000**
Log IL10 (n=15)	**0.895**	**0.000**	**0.979**	**0.000**	**-0.713**	**0.000**

Correlation analysis between variables was performed with the Spearman rank correlation, considering the complete set of four time points. Statistical significance was set at a $p < 0.05$.

Table 15: Correlations between the several types of variables with the apoptotic index and necrosis scores for all groups.

Variables	*Apoptotic Index (n=37)*				*Necrosis Score (n = 37)*	
	Cortical (n =37)		*Medula (n =37)*			
	Spearman r	*p value*	*Spearman r*	*p value*	*Spearman r*	*p value*
Hormonal						
CS (n=23)	**0.3043**	**0.160**	**0.1121**	**0.611**	**0.1327**	**0.547**
ACTH (n=23)	**0.2935**	**0.176**	**0.1178**	**0.593**	**0.3190**	**0.139**
Immunological						
IL6 (n=24)	**-0.1861**	**0.385**	**-0.1327**	**0.537**	**0.1314**	**0.541**
IL10 (n=24)	**0.2602**	**0.221**	**0.3947**	**0.058**	**0.6001**	**0.002**
TNF-*α* (n=24)	**-0.3289**	**0.118**	**-0.0583**	**0.787**	**0.3013**	**0.154**
Metabolic						
pH (n=34)	**-0.1329**	**0.454**	**-0.1210**	**0.496**	**-0.2704**	**0.122**
PO_2 (n=34)	**-0.0913**	**0.608**	**-0.1614**	**0.362**	**0.0551**	**0.757**
BE (n=34)	**-0.1867**	**0.291**	**-0.1739**	**0.326**	**-0.4401**	**0.009**
Lactate (n=20)	**-0.1872**	**0.431**	**-0.1555**	**0.514**	**0.2388**	**0.312**
Hemodynamic						
HR (n=13)	**0.569**	**0.047**	**-**	**-**	**0.726**	**0.007**
MAP (n=13)	**-**	**-**	**-**	**-**	**-**	**-**
Molecular Biology						
Log IL6 (n=10)	**0.6242**	**0.064**	**0.6429**	**0.055**	**0.5409**	**0.119**
Log IL10 (n=10)	**-0.4545**	**0.199**	**-0.7333**	**0.022**	**0.5409**	**0.119**
Log TNF-*α* (n=10)	**-0.4636**	**0.189**	**-0.6545**	**0.050**	**0.1782**	**0.627**

Correlation analysis between variables was performed with the Spearman rank correlation, considering the complete set of four time points. Statistical significance was set at a $p < 0.05$.